Characterization of TRAF6 Mediated Ubiquitination of Presenilins and γ-secretase Substrates

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by

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Declaration

This thesis has not been previously submitted, in part or in whole, to this or any other University for any degree and is, unless otherwise stated, the original work of the author.

Signed: _____

Run Yan

ABSTRACT

Post-translational modification of the γ-secretase protease complexes and their substrates has an important role in controlling receptor-initiated signalling events, which are critically important in the pathogenesis of cancer, inflammatory and Alzheimer's disease. Our lab has previously characterised an interaction between TRAF6 and presenilin-1, which lead to the identification of interleukin-1 (IL-1) receptor type 1 (IL-1R1) (Elzinga et al., 2009b) and Toll-like receptor-4 (TLR4) as novel γ-secretase substrates. Subsequently our group showed that TRAF6 promoted ubiquitination and γ-secretase cleavage of IL-1R1 (Twomey et al., 2009).

The aim of this project is to study the association between TRAF6 and the presenilins, the critical γ-secretase complex components, and to determine the functional importance of TRAF6-mediated ubiquitination of γ-secretase substrates. Firstly, we show that the full-length presenilins are novel substrates of TRAF6-mediated Lysine-63-linked polyubiquitination. Secondly, we show that co-expression of TRAF6 and the presenilins increases the stability and alters the turnover of the presenilins. Thirdly, we reveal that TRAF6-mediated ubiquitination of presenilin does not affect γ-secretase enzyme activity, but may regulate the full-length presenilin functions such as ER Ca^{2+} signalling.

Previously, we have reported IL-1R1 as a novel substrate of TRAF6-mediated ubiquitination. In this study, we identified five lysine residues in the IL-1R1 intracellular domain targeted by TRAF6-mediated polyubiquitination. Furthermore, mutagenesis of

these five lysine residues led to decreased IL-1R1 cell surface expression, precluded the ectodomain shedding and attenuated the responsiveness to IL-1β stimulation, demonstrating the critical role of TRAF6 in IL-1R1 trafficking.

Abbreviations

AD: Alzheimer’s disease

AICD: APP intracellular domain

APH-1: anterior pharynx-defective-1

APP: Amyloid precursor protein

ATP: Adenosine-5'-triphosphate

Aβ: Amyloid β

BACE1: β-site APP-cleaving enzyme 1

C99: 99-amino acid APP C-terminal fragment

cdk5/p35: cyclin-dependent kinase-5/p35

CK-1: casein kinase 1

CK-2: casein kinase 2

CTD: C-terminal domain

CTF: C-terminal fragment

CUE: coupling of ubiquitin conjugation to ER degradation

DN: dominant negative

DNA: Deoxyribonucleic acid

DUB: deubiquitination enzyme

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

E3: ubiquitin ligase

ER: Endoplasmic reticulum

FAD: Familial Alzheimer's disease

FL: full length

GSK-3: Glycogen synthase kinase-3

HBSS: Hanks buffered salt solution

ICD: Intracellular domain

IgG: Immunoglobulin G

IKK: inhibitor of kappa-B kinase

IL-1: Interleukin-1

IL-1RAcP: Interleukin-1 receptor accessory protein

IL-1R1: Interleukin-1 receptor type 1

LPS: lipopolyssacharides

IP: Immunoprecipitation

IRAK: IL-1 receptor associated kinase

IκB-α : inhibitor of NF-κB family

JNK: Jun N-terminal kinase

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinase

MEF: murine embryonic fibroblast

MKK: MAP kinase kinase

MyD88: Myeloid differentiation factor 88

NEMO: NF-κB essential modifier

NF-κB: nuclear factor-kappa B

NICD: Notch intracellular domain

Nrg1: neuregulin-1

NTF: N-terminal fragment

p75 NTR: p75 neurotrophin receptor

PEN-2: presenilin enhancer-2

PKC: Protein kinase A

PKC: Protein kinase C

PS1: Presenilin 1

PS2: Presenilin 2

RANK: receptor activator of nuclear factor-κB

RANKL: receptor activator of nuclear factor-κB ligand

RING: Really interesting new gene

sAPP: soluble APP

TAB1: TAK1 binding protein-1

TAB2: TAK1 binding protein-2

TAB3: TAK1 binding protein-3

TAK1: transforming growth factor β-activated kinase 1

TLR4: Toll-like Receptor 4

TMD: transmembrane domain

TNF-α: Tumour necrosis factor-α

TNFR: Tumour necrosis factor receptor

TRAFs: Tumour necrosis factor receptor-associated factors

UBD: ubiquitin-binding domain

Chapter 1:

INTRODUCTION

This section has been published in part as a review by Current Signal Transduction Therapy. [Yan, R. and McCarthy, J.V. (2009). Presenilin and gamma-secretase activity: a viable therapeutic target for Alzheimer's disease? Current Signal Transduction Therapy, Volume 5, Number 2, May 2010, pp. 128-140(13).]

1.1 Alzheimer's disease and APP cleavage

Alzheimer's disease (AD) is pathologically characterised by the formation and deposition of amyloid-β (Aβ) peptides in neuritic plaques in specific brain regions and cerebral vasculature. Aβ peptides are generated following the sequential proteolytic cleavage of amyloid precursor protein (APP) by the proteases, β-secretase and γ-secretase (Haass and Selkoe, 1993; Citron et al., 1995; Nunan and Small, 2000) **(Figure 1.1)**. APP is first cleaved by β-secretase releasing the larger soluble APP ectodomain (sAPPβ) and generating the amino terminus of Aβ peptides in the form of a membrane spanning 99-amino acid APP C-terminal fragment (CTF), APP C99. The membrane-anchored C99 is subsequently cleaved by γ-secretase at two locations: the ε-site to liberate cytosolic APP intracellular domain (AICD) and the γ-site to generate heterogeneous N-termini of Aβ-peptides (Weidemann et al., 2002). For this reason, both β- and γ-secretase are considered prime targets for the pharmacological reduction of APP cleavage and formation of Aβ peptides in the prevention and treatment of AD (Ghosh et al., 2005; Ghosh et al., 2008; Wolfe, 2008b, a). However, an important fact which should be kept in consideration is that in excess of one hundred type I membrane proteins including Notch, a critical receptor which is to determine in cell fate, are also cleaved by β- and γ-secretase (McCarthy et al., 2009a). So any potential treatment for reducing Aβ production should selectively inhibit APP cleavage, but not cleavage of Notch or other substrates.

The major β-secretase is a membrane anchored aspartyl protease of the pepsin family, termed BACE1 (β-site APP-cleaving enzyme 1), which is primarily expressed in the brain (Vassar et al., 1999; Luo et al., 2001). Biochemical and genetic studies have revealed that antagonising β-secretase activity is associated with mild phenotypes (Harrison et al., 2003; Ohno et al., 2004; Willem et al., 2006) and has therefore emerged as an attractive therapeutic target for the development of AD therapies (Ghosh et al., 2002; Ghosh et al., 2008). However, recent studies showed that BACE1 is required for the accurate targeting of olfactory sensory neuron axons and the proper formation of glomeruli in the olfactory bulbs and BACE1 deficiency causes spontaneous and pharmacologically-induced seizure activity (Hitt et al., 2010; Rajapaksha et al., 2011). Additionally, neuregulin-1, which is a critical factor in the development of the nervous system, has been shown as a substrate of BACE1 dependent proteolysis, underscoring the importance of substrate selectivity when inhibiting β-secretase activity for potential AD therapies (Vartanian et al., 1999; Fleck et al., 2011). Subsequently, the extracellular catalytic domain of β-secretase was crystallised (Hong et al., 2000; Hong et al., 2002), facilitating structure-based inhibitor design and accelerating the transition of β-secretase inhibitors into clinical trials, reviewed in (Ghosh et al., 2008; Ghosh et al., 2012). In contrast to β-secretase, the protease responsible for γ-secretase activity is a complex of four different integral membrane proteins: presenilin, Nicastrin, anterior pharynx defective 1 (Aph-1) and presenilin enhancer 2 (Pen-2) (Tolia and De Strooper, 2008).

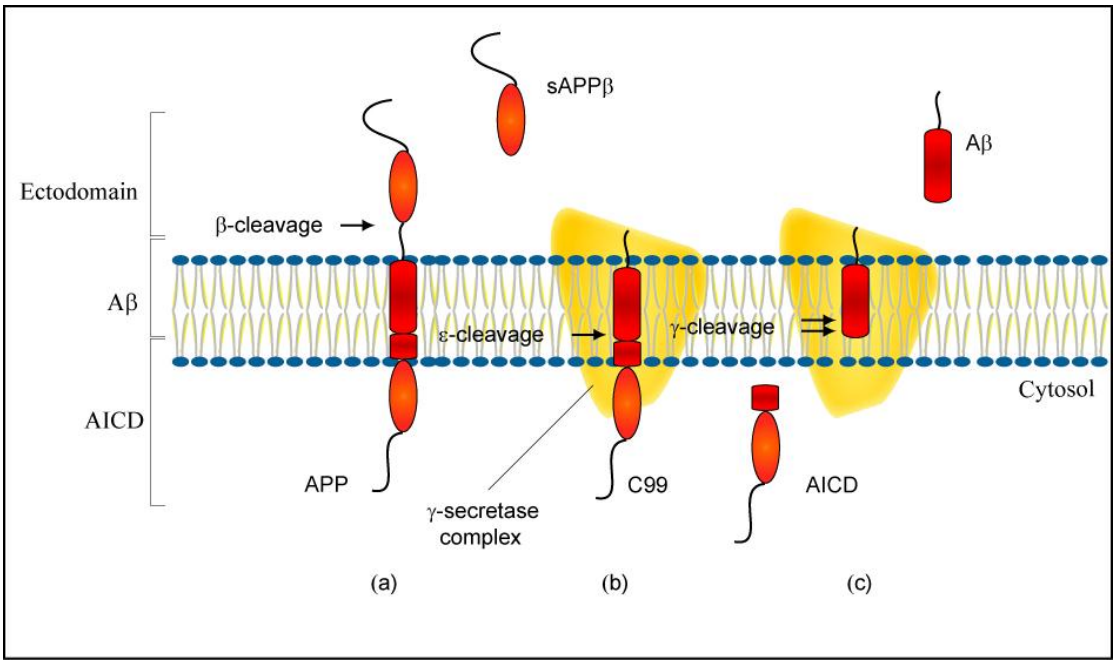


Figure 1.1 Schematic of APP cleavage. In this model the progressive proteolytic cleavage of amyloid precursor protein (APP) is illustrated. Firstly (a), cleavage in the APP ectodomain by β-secretase produces soluble ectodomain (sAPPβ) and the membrane-anchored C99 carboxyl-terminal domain. Next (b), the γ-secretase complex is recruited to APP-derived C99 and cleaves at the ε-cleavage-site to liberate the APP intracellular domain (AICD). Finally (c), γ-secretase cleaves the remaining membrane-associated fragment at the γ-cleavage site to liberate Aβ peptide fragments.

Furthermore, while β-secretase has few reported *in vivo* substrates (Sinha et al., 1999; Yan et al., 1999; Willem et al., 2006), subsequent to the identification of γ-secretase and its association with the proteolytic cleavage of APP, several independent studies have linked γ-secretase protease activity with the cleavage of over one hundred other substrates (Boulton et al., 2008; Lleo, 2008; McCarthy et al., 2009b) raising concerns about the selectivity, adverse toxicity and the overall validity of γ-secretase as a viable therapeutic target in the prevention and treatment of AD .

1.2 Gamma-secretase protease complexes

The initial discovery of two related genes (*PSEN1* and *PSEN2*) encoding the presenilin-1 (PS1) and presenilin-2 (PS2) proteins, and their association with autosomal dominant forms of familial AD (FAD) (Hutton and Hardy, 1997) has led to significant breakthroughs in our understanding of disease pathogenesis and development of potential AD therapeutics. To date more than 160 mutations in *PSEN1* and 10 mutations in *PSEN2* have been linked to early onset FAD (http://www.molgen.ua.ac.be/ADMutations). Most Aβ peptides range from 39 to 43 residues in length. Aβ42 and Aβ40 are composed of 42 and 40 amino acid residues respectively, of which toxic Aβ42 aggregates are considered critical in causing AD (Wang et al., 1996). Though the mutations occur throughout the presenilins they all cause a shift in the ratio of $A\beta_{42}$:$A\beta_{40}$ peptides resulting in a general increased accumulation of the more amyloidogenic $A\beta_{42}$ peptide (De Strooper, 2007). Genetic knockout of *PSEN1* in mice provided the first evidence that PS1 was absolutely

required for γ-secretase activity where it was demonstrated that *PSEN1*-deficency dramatically reduced production of Aβ peptides with a concomitant accumulation of the γ-secretase substrate, γ-secretase generated APP C99 fragments (De Strooper et al., 1998). Later reports demonstrated that knockout of both *PSEN1* and *PSEN2* resulted in complete ablation of γ-secretase activity and inhibition of Aβ peptide generation (Herreman et al., 2000). Some groups proposed that familial AD is caused by a partial loss of presenilin function because most of the AD-associated mutations in presenilins also cause a reduction in overall proteolytic activity (Song et al., 1999; Moehlmann et al., 2002; Schroeter et al., 2003; Bentahir et al., 2006). However, there are arguments showing that complete loss-of-function mutations in PS1, Pen-2 and Nicastrin in humans cause familial skin disorder but not AD (Wang et al., 2010) and AD mutations have only been found in APP but not any other γ-secretase substrate, suggesting that altered proteolytic cleavage of APP and shifted $A\beta_{42}$:$A\beta_{40}$ ratio are the critical factors for AD development.

In independent biochemical studies aimed at purifying presenilin-associated γ-secretase activity, PS1 and PS2 consistently co-purified with γ-secretase activity as part of larger high molecular weight multi-protein complexes (Li et al., 2000a; Li et al., 2000b; Esler et al., 2002; Kimberly et al., 2003), suggesting that PS1 and PS2 required additional cellular co-factors for biological and protease-associated functions. Subsequent high-grade biochemical purification studies and genetic screens lead to the identification and characterisation of three such co-factors, Nicastrin, APH-1 and PEN-2 (Tolia and De Strooper, 2008). The unconditional requirement for each of

these four integral membrane proteins for γ-secretase activity was verified following genetic ablation or RNAi knockdown of one or the other of the components, and genetic reconstitution of γ-secretase activity in *Saccharomyces cerevisiae*, which lack endogenous γ-secretase protease components (Edbauer et al., 2003; Tolia and De Strooper, 2008). All four proteins associate with each other and their co-expression results in increased γ-secretase activity in *Drosophila*, mammalian cells and reconstituted activity in yeast (De Strooper, 2003; Edbauer et al., 2003; Kimberly et al., 2003). This new multi-protein identity of the γ-secretase protease was subsequently corroborated by several studies reporting the purification of the active γ-secretase protease complex (Fraering et al., 2004a; Fraering et al., 2004b; Wakabayashi et al., 2009; Winkler et al., 2009; Teranishi et al., 2010). Presenilins are reported as the catalytic core of γ-secretase complex and two highly conserved aspartate residues in the transmembrane domain 6 and 7 (D257 and D385 in PS1, D263 and D366 in PS2) are essential for the endoproteolysis of presenilin and the proteolytic activity of γ-secretase (Steiner et al., 1999c; Wolfe et al., 1999). Additionally, γ-secretase inhibitors were shown to directly bind to the heterodimers of presenilin, providing evidence that presenilins contain the active sites of γ-secretase (Esler et al., 2000).Although presenilins are considered as the catalytic core of γ-secretase, recent studies showed the importance of the incorporation of the other subunits. Immature γ-secretase complex containing presenilin were suggested to have a relatively open conformation within the hydrophilic pore which was rendered a narrower pore structure that enables the enzymatic activity of γ-

secretase after the recruitment of the other subunits (Takeo et al., 2012). The activity of γ-secretase complex is also regulated by its adaptor proteins including the notable γ-secretase activating protein which interacts with both γ-secretase and APP CTF and confers substrate specificity to APP cleavage, providing a potential target for Alzheimer's disease therapy (He et al., 2010).

It is now generally well accepted that the γ-secretase protease complex consists of the four integral membrane proteins, presenilin, nicastrin, APH-1 and PEN-2 in a stoichiometry of 1:1:1:1 (Sato et al., 2007; Wakabayashi et al., 2009). However, in all examined species there are two *PSEN* genes (*PSEN1* and *PSEN2*), while in humans there are two *Aph-1* genes, *Aph-1a* and *Aph-1b*, which are alternatively spliced, and in rodents gene duplication of *Aph-1b* produces a third gene, *Aph-1c* (Hebert et al., 2004). Together with biochemical studies demonstrating that PS1 and PS2, or APH-1a and APH-1b/c, never coexist in the same γ-secretase protease complex, the potential existence of at least six distinct γ-secretase complexes in humans is proposed (Shirotani et al., 2004; Ma et al., 2005; Shirotani et al., 2007). Firstly, the presenilins and APH-1 proteins display different tissue distribution patterns. Secondly, PS1-deficient and PS2-deficient mice have dramatically different phenotypes suggesting a diversity of presenilin-associated cellular functions and incomplete functional redundancy. Indeed, biochemically purified PS1-associated γ-secretase complexes have higher specific activity (>150 fold) than PS2-associated γ-secretase complexes (Lai et al., 2003; Yonemura et al., 2011), and both are differentially antagonised by pharmacological inhibitors of γ-secretase activity.

Thirdly, from phenotypic characterisation of *Aph-1* deficient mice, differential contribution of the *Aph-1* genes (*Aph-1a* or *Aph-1b/c*) to γ-secretase activity have been reported (Serneels et al., 2005; Dejaegere et al., 2008) where the APH-1a-containing γ-secretase complexes appear to be critical for Notch signalling while APH-1b/c-containing γ-secretase complexes are dispensable for Notch signalling during embryogenesis. In contrast deficiency of APH-1b/c-containing γ-secretase complexes antagonises cleavage of the γ-secretase substrate neuregulin-1 (Nrg1), but not other substrates (ErbB4, Syndecan and Notch,) (Dejaegere et al., 2008), suggesting brain-specific function for APH-1b/c-containing γ-secretase complexes. Indeed, it has now been clearly demonstrated that different APH-1-contaning γ-secretase complexes have heterogeneous biochemical and physiological properties (Serneels et al., 2009). Specifically, APH-1b-containing γ-secretase complexes contribute to total γ-secretase activity in the human brain, while inactivation of the APH-1b in a murine AD model contributes to improvements of AD-relevant phenotypic features without any apparent Notch-related side effects (Serneels et al., 2009).

1.3 Non-proteolytic function of presenilins

In the brain, presenilins are predominantly present as NTF and CTF heterodimer which are reported to be generated in the endoplasmic reticulum (ER) and incorporated into the γ-secretase complex during the its transport from the ER to the Golgi apparatus (Spasic et al., 2006a). However, Presenilin functions independent of γ-secretase complex are also being studied which contributes to the understanding

between presenilin functions and AD pathogenesis. To verify a presenilin function is γ-secretase independent, the phenotype in presenilin deficient cells should be rescued by the endoproteolytically inactive presenilin mutants and not mimicked by γ-secretase inhibitors.

Earlier and recent studies both showed that full-length presenilins are localised predominantly in the ER (Walter et al., 1996b; Area-Gomez et al., 2009). Consistent with this observation, full-length presenilins are reported as passive ER Ca^{2+} leak channels and this function is disrupted by many FAD mutations (Tu et al., 2006; Nelson et al., 2007; Nelson et al., 2010; Zhang et al., 2010; Nelson et al., 2011). Full-length presenilins alone are capable to exert ER Ca^{2+} signalling function and deficiency of the ER Ca^{2+} signalling in the presenilin double knockout (DKO) cells could be rescued by presenilin aspartate mutations revealing a γ-secretase independent full-length presenilin function (Brunello et al., 2009). Inducing ER stress by tunicamycin elevates PS1 transcription which could confer resistance to ER stress by forming complex of PS1 holoprotein and sarco ER calcium-ATPase channel, thus regulating intracellular Ca^{2+} homeostasis (Jin et al., 2010). Similarly, inhibition of c-jun-NH2-terminal kinase (JNK) activation represses *PSEN1* transcription and leads to impaired ER Ca^{2+} leak function which is also observed with FAD mutations (Das et al., 2012). Because altered Ca^{2+} release was detected from a large proportion of AD family members prior to the development of their AD symptoms but not in the family members who failed to develop AD (Etcheberrigaray et al., 1998), late studies then aimed to explore the role of FAD presenilin mutations and revealed the

deficiency in ER Ca^{2+} signalling caused by the many FAD mutations which leads to the proposal of the "calcium hypothesis". The calcium hypothesis attempts to explain altered Ca^{2+} signalling of AD either as results of Aβ oligomerisation which acts as or activates plasma membrane channels to increase Ca^{2+} entry, or as the down-stream signalling events of APP ICD which may alter the expression of the key ER components such as ryanodine receptor (Berridge, 2010; Zhang et al., 2010). Knock-out of presenilins or presenilin FAD mutations has also been shown to associate with lysosomal defects including autophagic deficits and abnormal lysosomal acidification (Lee et al., 2010; Neely et al., 2011). Some groups proposed that lysosomal fusion events may require Ca^{2+} release from lysosome and reduced Ca^{2+} loading into lysosomes which causes lysosomal abnormalities may potentially result from impaired ER Ca^{2+} leak function in presenilin DKO or FAD cells (Bezprozvanny, 2012). Supporting this hypothesis, one study showed that content of lysosomal Ca^{2+} stores was significantly reduced in PS DKO MEF cells and in PS1 KO neurons which could be rescued by stable retroviral transduction with PS1 or γ-secretase activity defective PS1 mutant (Coen et al., 2012).

Another γ-secretase-independent presenilin function has been reported as its interaction with and regulation of β-catenin and β-catenin mediated cell adhesion. β-catenin is targeted for constitutive degradation mediated by protein kinase A (PKA) and glycogen synthase-3β (GSK-3β) which requires a scaffold complex comprised of Axin and APC (Huang and He, 2008). Wnt signalling blocks phosphorylation by GSK-3β, thus preventing β-catenin degradation and allowing its nuclear translocation.

Presenilin was initially identified as a negative regulator of β-catenin function (Cox et al., 2000; Noll et al., 2000). Follow-up studies either proposed presenilin as a suppressor of Wnt-mediated stabilization and nuclear translocation of β-catenin (Killick et al., 2001), or as an alternative scaffold for β-catenin phosphorylation after Axin is targeted for degradation during Wnt signalling (Kang et al., 1999; Soriano et al., 2001; Kang et al., 2002). Notably, PS1 D257A mutant, defective in γ-secretase activity, restored β-catenin turnover as same as the wild-type PS1, revealing a γ-secretase-independent presenilin function (Kang et al., 2002). β-catenin also functions to link the cadherins to α-catenin which enables the formation of a linkage between adherence junctions and the cytoskeleton, thus stabilize intercellular adhesions (Parisiadou et al., 2004). Expression of PS1 has been shown to stabilize the E-cadherin/β-catenin/α-catenin complex and absence of PS1 results in decreased complex and cellular adhesion (Baki et al., 2001). Consistent with this, abnormalities in the cytoskeleton within a kind of presenilin inactivated moss was also observed, which could be rescued by human presenilin Asp-mutations (Khandelwal et al., 2007). However, other studies suggested that presenilin regulates β-catenin mediated cell adhesion and signalling through γ-secretase mediated cleavage of leukocyte-common antigen-related (LAR) receptor tyrosine phosphatase which argued that presenilin mediated β-catenin regulation is γ-secretase dependent (Haapasalo et al., 2007). Similar to the regulation of β-catenin mediated cell adhesion, loss of presenilins also leads to enhanced maturation and cell-surface delivery of mature integrin β1, resulting in increased cell adhesion which could not be mimicked by γ-

secretase inhibitors and presenilin was suggested to inhibit the maturation of integrin β1 in the ER (Zou et al., 2008).

Presenilins have also been implicated in protein trafficking presumably through its association with several vesicle transport proteins, for example, syntaxins (Smith et al., 2000; Suga et al., 2004)or members of the Rab family of small GTPase proteins (Dumanchin et al., 1999; Kametani et al., 2004; Scheper et al., 2004). One well studied non-proteolytic function of presenilins in trafficking is their role in the turnover of autophagic vacuoles. Deficiency of PS1 or presenilin DKO led to telencephalin (TLN or ICAM-5) and α- and β-synuclein abnormal aggregation respectively, resulting in failed fusion of autophagic vacuoles with the endosome/lysosome thus affecting autophagic vacuoles maturation (Annaert et al., 2001; Wilson et al., 2004). The abnormal trafficking of TLN in PS1 deficient neurons was rescued by expression of wild-type PS1 or PS1 D256A and was not mimicked by γ-secretase inhibitors, indicative of a γ-secretase independent function of presenilin (Esselens et al., 2004). In another case, deficiency of PS1 or expression of some FAD PS1 mutants led to impaired kinesin-I-mediated fast axonal transport which was observed with increased GSK-3β activity (Pigino et al., 2003; Lazarov et al., 2007). GSK-3β phosphorylates kinesin light chains and causes the dissociation of kinesin-I from membrane-bound organelles, resulting in impaired kinesin-I-mediated transport (Morfini et al., 2002). PS1 was shown to inactivate GSK-3β by promoting the phosphorylation of GSK-3β through the PI3K/Akt signalling in a γ-secretase

inhibitor insensitive manner, thus proposed to regulate kinesin-I-mediated transport indirectly (Baki et al., 2008).

1.4 Transcriptional regulation of *PSEN1* and *PSEN2*

Endogenous transcription of presenilins is tightly regulated which maintains relatively stable levels of presenilin protein. Increasing the levels of PS1 protein decreases normal *PSEN1* transcription and blockage of PS1 translation increases *PSEN1* transcription (Thinakaran et al., 1997; Nornes et al., 2008; Newman et al., 2012). Mutations in PS1 promoter have also been implicated with increased risk of developing AD (Theuns et al., 2000; Lambert et al., 2001; Theuns et al., 2003). Expression of *PSEN1* is activated by a variety of transcriptional activators including Ets, Ets related molecule (ERM), 12-O-tetradecanoylphorbol 13-acetate (TPA) and cAMP-response element-binding protein (CREB) (Mitsuda et al., 2001; Pastorcic and Das, 2002, 2004, 2007a). Some of these transcriptional activators also bind to their repressor proteins, for example p53 interacts with Ets1/2 and ZNF237 and CHD3/ZFH bind to ERM, all leading to suppressed PS1 transcription (Pastorcic and Das, 2007a, b; Lee and Das, 2008). In addition, human *PSEN2* promoter is modulated by transcription factor Egr-1 (Ounallah-Saad et al., 2009). AICD has been shown to interact with p53 and enhance its transcriptional and pro-apoptotic functions (Ozaki et al., 2006). Inhibition of JNK activation represses PS1 transcription by losing p-JNK inhibiting effect on p53 activity (Lee and Das, 2008). Transcription of PS1 is also triggered by intracellular stimuli events. Firstly, ER stress induced by tunicamycin increases PS1 expression and results in enhanced γ-secretase activity and ER Ca^{2+}

signalling ability (Jin et al., 2010; Ohta et al., 2011). Moreover, ethacrynic acid induced oxidative stress increases PS1 expression, causes enhanced PS1 protein levels in lipid rafts and results in higher Aβ secretion (Oda et al., 2010). Additionally, knock-down of the purine reutilization enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) leads to reduced PS1 expression and concomitant altered PS1 fragment levels (Kang et al., 2011). Collectively, there appears to be a close loop control between *PSEN* transcription and its functions where altered presenilin function leads to disrupted cellular homeostasis (altered AICD production, ER stress or oxidative stress) which consequently causes adjustment in presenilin transcription thus feeding back to presenilin functions.

1.5 Presenilin post-translational modification

Full-length presenilins are synthesized as inactive holoproteins which are subsequently endoproteolytically cleaved producing active presenilin NFT/CTF heterodimers (Thinakaran et al., 1996). Posttranslational modifications of presenilins which includes proteolysis, phosphorylation and ubiquitination are not only essential for the stability and activation of presenilins, but are also important for the formation and activity of γ-secretase complexes.

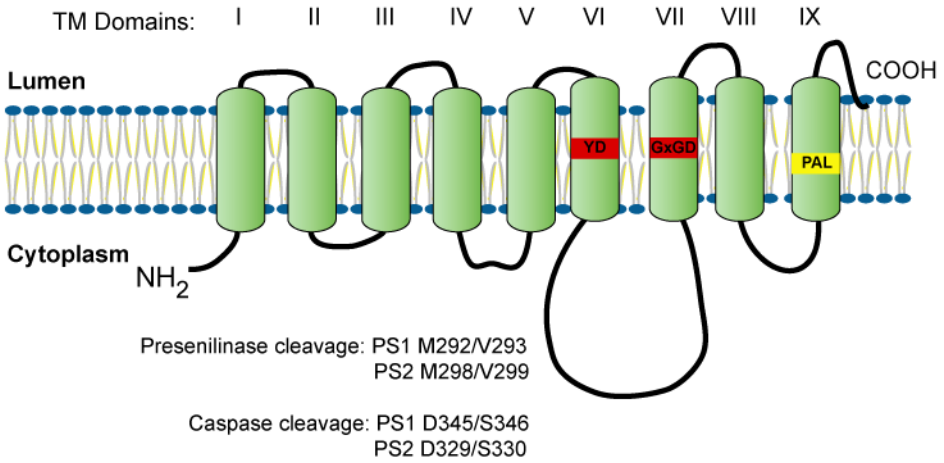
1.5.1 Endoproteolysis and caspase cleavage

The presenilin proteins are multi-transmembrane proteins consisting of nine transmembrane domains (TMD), which are endoproteolytically cleaved into a ~30-kDa NH_2-terminal fragment (NTF) and a ~20-kDa COOH-terminal fragment (CTF). Due

to the short half-life of presenilin holoproteins (approximately 1h) and comparably long half-life of presenilin fragments (~24h), NTF/CTF heterodimers are the major presenilin species *in vivo*. The endoproteolytic event occurs between residues Met292/Val293 and Met298/Val299 within the large cytosolic loop in human PS1 and PS2 and stable NTF/CTF complexes are formed (Thinakaran et al., 1996; Jacobsen et al., 1999) **(Figure 1.2)**. The NTF/CTF heterodimers comprise the catalytic component of γ-secretase (Kopan and Ilagan, 2004), and two active site aspartate residues are located in each fragment, Asp257 in the NTF (TMD6) and Asp385 in the CTF (TMD7) in human PS1 (Wolfe et al., 1999), Asp263 and Asp366 in human PS2 (Steiner et al., 1999b). Mutations of these conserved aspartate residues abolish γ-secretase activity and presenilin endoproteolysis but not affect the expression of presenilin or the formation of the γ-secretase complex (Yu et al., 2000; Nyabi et al., 2003). The presenilins are endoproteolysed by an unknown protease, presenilinase, which is assumed to be γ-secretase or presenilin itself because mutations of the catalytic aspartate residues abolish presenilin proteolysis. Although it has not been formally proven, the presenilins are proposed to undergo an autoproteolytic event that activates itself to form the active NTF/CTF heterodimers (Li et al., 2000b; Brunkan et al., 2005; Fukumori et al., 2010). A human PS1 Exon 9 deletion mutant (PS1ΔE9), in which the putative endoproteolysis site is removed, is able to induce a structural change that stabilizes the uncleaved full-length PS1 and perform biological and pathological function suggesting the implicated activity of full-length presenilins independent of the γ-secretase activity (Steiner et al., 1999a).

Figure 1.2 Presenilin structure, functional domains and potential sites of posttranslational modification. (A), Schematic representation of presenilin-1 (PS1) structure. PS1 contains ten hydrophobic domains arranged in a predominant nine-transmembrane domain (TMD) topology with a large hydrophilic loop domain between TMD six and seven. The protease activity of γ-secretase complexes is mediated by two aspartyl protease active site motifs (YD and GxGD) located in the centre of adjacent TMD 6 and TMD 7 with reverse orientation, and the PAL motifs located in TMD 9. The cytosolic loop contains the Presenilinase and caspase cleavage sites, mapped as indicated. (B) Previously described covalent posttranslational modifications of human presenilins, PS1 and PS2. PS1 contains three GSK3β phosphorylation sites (underlined) whereas PS2 contains none. PS1 is subject to serine/threonine phosphorylation by PKA (●), PKC (○), CDK5 (■), GSK3β (□) and JNK (◊) and PS2 is subject to serine/threonine phosphorylation by CK1/2 (∗) and mapped to specific individual residues. PS1 is also ubiquitinated by Sel-10, but the site(s) have not been mapped to individual residues. The endoproteolysis and caspase cleavage sites are also indicated (arrows).

A



B

Human Presenilin-1

281 TLFPALIYSSTMVWLVNMAEGDPEAQRRVSKNSKYNAESTERESQDTVAENDDGGFSE

339 EWEAQRDSHLGPHRSTPESRAAVQELSSSILAGEDPEERGVKLGLGDFIFYSVLVGKA

397 SATASGDWNTTIACFVAILIGLCLTLLLLAIFKKALPALPISITFGLVFYFATDYLVQ

Human Presenilin-2

277 LVETAQERNEPIFPALIYSSAMVWTVGMAKLDPSSQGALQLPYDPEMEEDSYDSFGEP

333 SYPEVFEPPLTGYPGEELEEEEERGVKLGLGDFIFYSVLVGKAAATGSGDWNTTLACF

393 VAILIGLCLTLLLLAVFKKALPALPISITFGLIFYFSTDNLVRPFMDTLASHQLYI

Another study demonstrates that Presenilinase-mediated endoproteolysis of presenilins is not essential for the maturation of presenilins in *Drosophila* (Barakat et al., 2009). Endoproteolysis of the presenilin NTF/CTF appears to be tightly regulated. The levels of full-length PS1 increase corresponding to the levels of expressed mRNA encoding human PS1, but no increase is observed in corresponding human PS1 NTF and CTF. Furthermore, overexpression of the presenilin proteins does not result in a parallel increase of fragments formation indicating that additional proteins are required for presenilins to mature into stable NTF/CTF heterodimers (Thinakaran et al., 1996). Another study shows that overexpression of ubiquilin, which interacts with presenilins, decreases presenilin NTF and CTF levels (Massey et al., 2005). Phosphorylation by GSK-3β, protein kinase C (PKC) and PKA in PS1 CTF hydrophilic loop domain also regulate the level of PS1 CTF (Kirschenbaum et al., 2001a). Presenilin NTF/CTF generated by endoproteolysis is indispensable components of the γ-secretase complex, and the fragments are also important for the maturation and stability of presenilin complex.

In addition to the endoproteolysis, presenilins also undergo caspase-dependent cleavage. Several groups have reported the caspase-dependent cleavage sites revealed by N-terminal protein sequencing that human PS2 is endoproteolyted between Asp329 and Ser330 (Loetscher et al., 1997), and human PS1 is cleaved between Asp345 and Ser346 (Grunberg et al., 1998) **(Figure 1.2B)**, with both sites conforming to the consensus caspase-recognition sites. Phosphorylation of PS1 at Ser346 by PKC *in vitro and in vivo* inhibits this caspase-dependent cleavage (Fluhrer

et al., 2004). Similarly, PS2 is phosphorylated by the second messenger-independent casein kinase 1 (CK-1) and/or casein kinase 2 (CK-2) at serine 327 and 330, which inhibits the caspase-dependent cleavage *in vitro and in vivo* (Walter et al., 1996a; Walter et al., 1999). Phosphorylation of proteins at caspase recognition sites modulates the progression of apoptosis, and it has been suggested that the caspase-cleaved PS1 CTF promotes apoptosis (Kim et al., 1997a; Loetscher et al., 1997). Co-expression of PS1-NTF and caspase-cleaved PS1 CTF reconstitute γ-secretase activity in presenilin-null cells (Hansson et al., 2006), indicating the redundancy of the N-terminus end of PS1 CTF. Furthermore, caspase-cleaved PS1 CTF containing γ-secretase complex was shown to exhibit increased production of Aβ42 and increased Aβ42/Aβ40 ratio (Hedskog et al., 2011). However another study reported that deletion of the hydrophilic loop containing the caspase cleavage sites in PS1 does not disrupt Aβ production (Saura et al., 2000), suggesting that alteration with the caspase cleavage of PS1 does not alter γ-secretase activity.

1.5.2 Phosphorylation of the presenilins

As demonstrated previously, human PS1 CTF is phosphorylated at Ser 346 by PKC *in vitro* and *in vivo*, whereas PKA phosphorylates human PS1 CTF exclusively at Ser310 (Fluhrer et al., 2004) **(Figure 1.2B)**. It has been suggested that caspase-cleaved PS1 CTF promotes apoptosis (Kim et al., 1997a; Loetscher et al., 1997) and PKC-mediated phosphorylation reduces the generation of caspase-cleaved PS1 CTF which might explain the inhibition effect on the progression of apoptosis. However, phosphorylation by PKA does not inhibit the caspase-dependent cleavage of PS1,

with the function of this phosphorylation remaining unknown (Fluhrer et al., 2004). The presenilins are also reported to regulate PKC levels and activity, which might be explained as the activation of the system to protect the presenilin proteins from caspase-cleavage (Dehvari et al., 2007). Human PS2 CTF is phosphorylated *in vivo* by CK-1 and/or CK-2 at Ser327 and Ser330 which are adjacent to the reported caspase-cleavage sites (Asp326 and Asp329). Phosphorylation of PS2 CTF also blocks caspase-dependent cleavage of PS2 CTF and interferes with its function in apoptosis. It was also demonstrated that cellular expression of phosphorylated PS2 CTF dramatically inhibits apoptosis (Walter et al., 1999). Considering the relevant properties in the caspase-dependent cleavage and apoptosis progression, phosphorylation of the presenilins at caspase recognition sites are suggested to provide a mechanism to protect the presenilins against caspase-dependent cleavage and stabilize the anti-apoptotic protein.

Given that the presenilins contain several putative phosphorylation sites, it is no surprise that the presenilin proteins have been shown to be phosphorylated by several other kinases. Firstly, PS1 is an unprimed substrate of glycogen synthase kinase 3β (GSK3β) and is phosphorylated at Ser353, Ser357, Ser397 and Ser410 by GSK3β (Kirschenbaum et al., 2001b; Twomey and McCarthy, 2006) **(Figure 1.2B)**. GSK3β-mediated phosphorylation of PS1 regulates its binding to N-cadherin. GSK3β-mediated phosphorylation also regulates APP cleavage by γ-secretase (Uemura et al., 2007). Another study has demonstrated that GSK3β-mediated phosphorylation induces a structural change of the hydrophilic loop of PS1 and reduces the

interaction of PS1 with β-catenin which leads to the reduction of β-catenin phosphorylation and ubiquitination and stabilizes β-catenin (Prager et al., 2007). Another GSK3β phosphorylation site (Ser397) within the loop domain of PS1 has also been identified, phosphorylation of which regulates the PS1 CTF levels (Kirschenbaum et al., 2001a). Secondly, PS1 is reported to be phosphorylated by cyclin-dependent kinase-5/p35 (cdk5/p35) at Thr354 within PS1 CTF both *in vitro* and *in vivo* (Lau et al., 2002) **(Figure 1.2B)**. Phosphorylation of cdk5/p35 is suggested to stabilize PS1 CTF selectively, therefore act as a regulator of PS1 metabolism. Similarly, PS1 was showed to be phosphorylated by the dual-specificity tyrosine(Y)-phosphorylation-regulated kinase 1A (Dyrk1A) also at Thr (354) which stabilizes PS1 and increases γ-secretase activity (Ryu et al., 2010).Moreover, another study identified a c-Jun N-terminal kinase (JNK) phosphorylation site within human PS1 at Ser319 and Thr320. This study shows that JNK-dependent phosphorylation of PS1 enhances the stability of PS1 CTF, and mediates tumour necrosis factor (TNF)-α-induced stimulation of γ-secretase (Kuo et al., 2008), suggesting that phosphorylation of PS1 by JNK might contribute to γ-secretase protease activity and therefore the pathogenesis of AD. Finally, recent study showed that phosphorylation of PS1 inhibits insulin receptor transcription and expression thus promotes down-regulation of insulin signalling which may be associated with AD pathology considering the role of insulin resistance as a risk factor for sporadic AD (Maesako et al., 2012).

1.5.3 Ubiquitination of presenilins

It is reported that, human SEL-10, a homologue of yeast Cdc4, a member of the Skp1-Cdc53/CUL1-F-box protein E2-E3 ligase family, interacts with human PS1, and enhances its ubiquitination. Furthermore, transfection of SEL-10 increases Aβ levels observed with the unexpected reduction of PS1 NTF and CTF levels, suggesting that SEL-10-mediated ubiquitination might regulate PS1 activity in APP processing (Li et al., 2002). Another earlier report shows that, elimination of SEL-10 activity leads to the functional reducing of SEL-12, a *C. elegans* presenilin homologue (Wu et al., 1998), which also suggests that SEL-10 regulates presenilin levels and activities. Additionally, inhibition of phosphatidylinositol-3 kinase (PI3K) leads to the multiple mono-ubiquitination of PS1 and precludes PS1 degradation through the proteasomal pathway thus increases level of PS1 and alters its distribution (Aoyagi et al., 2010). Moreover, it has been shown that mutation of two lysine residues in PS2 reduces its ubiquitination, results in the destabilization of PS2 and inhibits its binding to ubiquilin demonstrating the importance of ubiquitination modification for the stability and activity of PS2 (Ford and Monteiro, 2007).

Presenilins undergo proteasome degradation after being modified by polyubiquitination (Kim et al., 1997b; Fraser et al., 1998; Marambaud et al., 1998). Ubiquilin is an important presenilin-interacting protein involving in proteasome degradation pathway of presenilins. Ubiquilin is proposed to interact with polyubiquitinated presenilin through its ubiquitin-associated (UBA) domain, thus inhibiting presenilin being targeted by proteasomal degradation and leading to accumulation of high molecular weight (polyubiquitinated) presenilin (Mah et al.,

2000; Massey et al., 2004). Mutations of PS2 which disables its binding to Ubiquilin result in destabilization of PS2 and increased degradation by proteasome (Ford and Monteiro, 2007). AD associated Ubiquilin transcript variant which lacks the proteasome-interaction domain also increases levels of full-length PS1 and high molecular weight PS1, accumulation of which leads to aggresome formation that is further targeted by autophagosome (Viswanathan et al., 2011). Inhibition of proteasome degradation or overexpression of presenilin both lead to chaperone-mediated formation of presenilin-containing aggresome which is considered as a general cellular response to misfolded protein that is removed by autophagocytosis (Johnston et al., 1998; Kovacs et al., 2006). Other proteins were also reported to involve in presenilin proteasomal degradation. Firstly, deletion of the ubiquitin-like domain of Herp inhibits the degradation of overexpressed full-length presenilin (Marutani et al., 2011). Secondly, inhibition of PI3K increases PS1 level by inducing multiple mono-ubiquitination of PS1 which precludes the degradation of PS1 through the proteasomal pathway (Aoyagi et al., 2010). Finally, knock-out of Nicastrin results in decreased PS1 fragments but accumulation of the full-length PS1 in the ER which is subjected to the proteasome-mediated degradation (Zhang et al., 2005).

All studies discussed above confirm a fact that the presenilins are subjected to posttranslational modifications which alter the presenilin functions and interactions with other proteins. Collectively, a variety of presenilin-dependent activities are modulated by posttranslational modification of presenilins. Firstly, caspase-

dependent cleavage of presenilins is inhibited by the PKC and CK-1/2 induced phosphorylation of the presenilin proteins (Walter et al., 1996a; Walter et al., 1999). Secondly, β-catenin stability and its nuclear signalling are facilitated by GSK3β-mediated phosphorylation through modulating the interaction between PS1 and β-catenin (Prager et al., 2007). Finally, turnover of PS1 and its fragments is affected by SEL-10-induced ubiquitination of PS1. Stability of PS1 CTF is altered by cdk5/p35 and JNK-dependent phosphorylation of PS1 (Lau et al., 2002; Kuo et al., 2008). Moreover, TNF-α-stimulated, JNK-dependent phosphorylation is suggested to mediate γ-secretase activity (Kuo et al., 2008), indicating a proposed linkage between presenilin posttranslational modifications and γ-secretase activity.

1.6 Protein ubiquitination

Ubiquitin is a highly conserved 76-amino-acid protein which is covalently attached to its ubiquitinated targets through an isopeptide bond between its C-terminal glycine and the lysine of the target protein (Vijay-Kumar et al., 1987). Four types of enzymes are involved in ubiquitination: E1-E4. First, a high-energy thioester bond is formed between E1 (ubiquitin-activating enzyme) and the C-terminal of ubiquitin in an ATP-dependent reaction. Then the activated ubiquitin is transferred to the cysteine residue of E2 (ubiquitin-conjugating enzyme). The E3 enzymes (ubiquitin-ligase) catalyses the formation of isopeptide bond with the lysine residue in the substrate proteins **(Figure 1.3)**. After the first ubiquitin is attached (monoubiquitination), E3

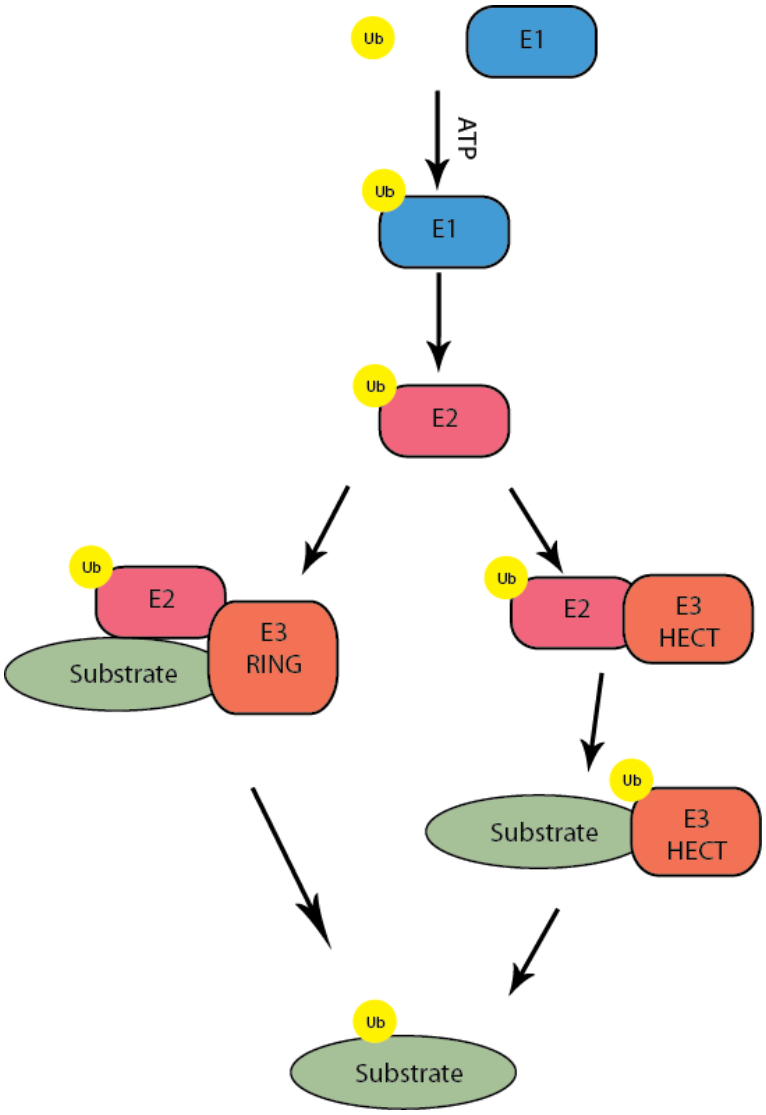


Figure 1.3 The ubiquitination pathway. Ubiquitin is activated in an ATP-dependent manner by an ubiquitin-activating enzyme (E1), and it is transferred to an ubiquitin-conjugating enzyme (E2). The RING domain ubiquitin-protein ligases (E3) interact with both E2 and the substrate and transfer the ubiquitin directly from E2 to the substrate. The HECT domain E3s firstly link to ubiquitin via E2 and then recruit the substrate and catalyse the substrate ubiquitination.

ligases can elongate the ubiquitin chain by creating ubiquitin-ubiquitin linkages through their lysine residues present in ubiquitin (polyubiquitination). The E4 enzymes (chain elongation factors) are a subclass of E3-like enzymes which only function in ubiquitin chain extension (Pickart, 2004; Ikeda and Dikic, 2008). In the human proteome, there are two ubiquitin E1s, ~40 E2s and ~600 E3s. All E2s contain an ubiquitin-conjugating domain of ~140 amino acids which has a cysteine residue at the active site. E3 ubiquitin ligases can be classified into two subfamilies: HECT (homology to E6AP C-terminus) domain E3s which contain a highly conserved cysteine residue, and RING (really interesting new gene) domain E3s. There is no classical active enzyme site in RING domain E3s, but they can bind to E2s and substrates and mediate the ubiquitination of substrates by E2s (Chen and Sun, 2009). Ubiquitin has seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63), all of which can be conjugated with another ubiquitin to form polyubiquitin chains of different linkages (Peng et al., 2003). Polyubiquitin chains linked through Lys48 of ubiquitin lead to degradation of the substrates by the 26S proteasome, whereas Lys63 chains perform not only degradation but also other biological functions including endocytosis, protein sorting, receptor trafficking and DNA damage repair (Pickart, 2001; Madura, 2002). Ubiquitins on substrates can also be disassembled by deubiquitination enzymes (DUBs) (Nijman et al., 2005), therefore ubiquitination and deubiquitination of target protein can act as a reversible modification.

At least 20 different ubiquitin-binding domains (UBDs) have been reported, and most of them bind to a hydrophobic region surrounding Ile44 of ubiquitin. CUE (coupling

of ubiquitin to ER degradation) domain is one of the UBDs which use α-helix to contact the Ile44 surface of ubiquitin (Kang et al., 2003; Prag et al., 2003). It is reported that CUE domain promotes monoubiquitination of proteins within which it is carried and may serve as a scaffold for interaction with E2 enzymes (Ponting, 2000a; Shih et al., 2003b).

1.7 TRAF family proteins

The TNF receptor-associated factor (TRAF) family of proteins have been shown to play an important role in several signalling pathway to activate the nuclear factor-kappa B (NF-κB) transcription factor and mitogen-activated protein kinases (MAPK) (Bradley and Pober, 2001; Chung et al., 2002b). Seven members of the TRAF family (TRAF1-TRAF7) have been identified so far **(Figure 1.4)**. They share a common structural domain, the C-terminal homology region, which is able to bind to the cytoplasmic domain of receptors and other TRAF proteins. All TRAF proteins, except TRAF1, also contain an N-terminal RING domain which is found in a number of E3 ubiquitin ligases, followed by several zinc fingers. The RING domain and zinc finger domains are essential for the activation of downstream signalling cascades (Chung et al., 2002b).

TRAF6 contains a C-terminal domain, an N-terminal domain, and a series of four zinc fingers which connects the N- and C-terminal regions. One study showed that the first zinc finger and an intact RING domain of TRAF6 are required for its functions in

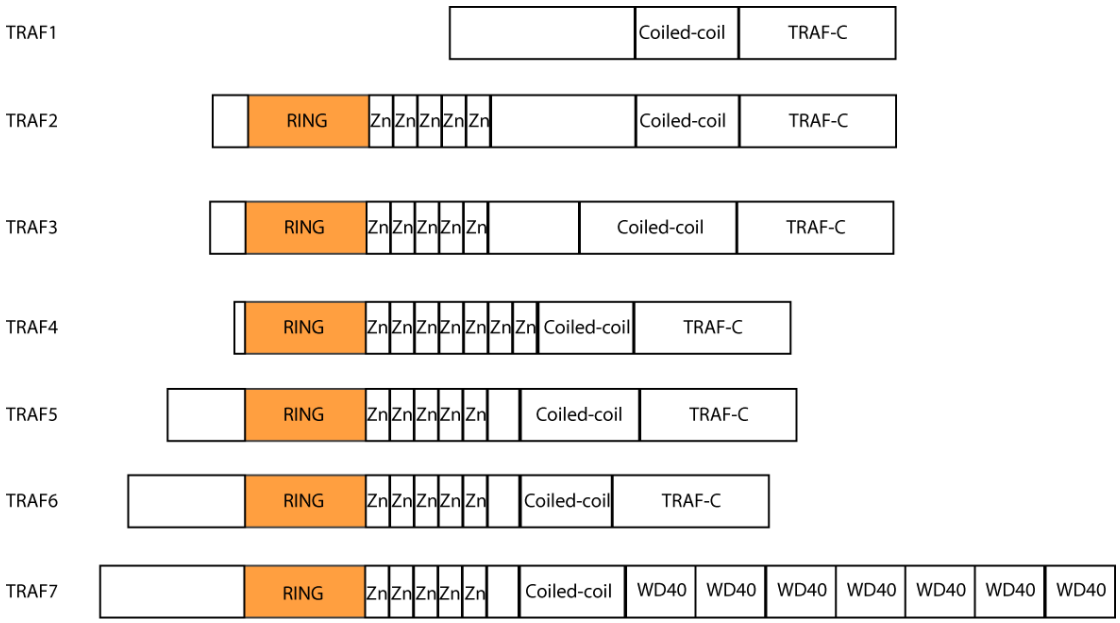


Figure 1.4 Domain structure of the TNF receptor-associated factor (TRAF) protein family. TRAF1, unlike other TRAF members, does not contain the N-terminal really interesting new gene (RING) and zinc-finger (Zn) domains. The C-terminal TRAF domain comprises of the coiled-coil domain and a highly conserved TRAF-C domain. TRAF7 is structurally different by identification of seven WD40 domains at its C-terminus instead of the TRAF domain.

interleukin-1 (IL-1), lipopolysaccharide (LPS) and receptor activator of nuclear factor-κB ligand (RANKL) signalling (Lamothe et al., 2008). In conjugation with a dimeric E2 enzyme complex (Ubc13-UeV1A), the RING domain of TRAF6 achieves E3 ubiquitin ligase activity to catalyse the formation of a poly-ubiquitin chain linked through Lys-63 of ubiquitin (Deng et al., 2000). Unlike Lys48-linked polyubiquitination, which targets proteins for proteasomal degradation, Lys63-linked polyubiquitination does not degrade targeted proteins, but activates signalling pathways.

TRAF6 can facilitate its own site-specific autoubiquitination by generating a Lys63-linked polyubiquitination chain (Deng et al., 2000) and also activate itself by Lys63-linked polyubiquitination. Autoubiquitination of TRAF6 leads to recruitment and activation of TAK1 (transforming growth factor β (TGFβ)-activated kinase 1) –TAB1 (TAK1-binding protein 1) –TAB2/3 complex, as TAB2/3 specifically recognizes Lys63-linked polyubiquitination chain on TRAF6 and recruits TAK1 to TRAF6 complexes **(Figure 1.5)**. Activated TAK1 complex then phosphorylates and activates inhibitor of κ-B kinase (IKK) and mitogen-activated protein kinase kinases (MKKs) and these in turn phosphorylates and activate NF-κB and MAPKs such like JNK and p38 (Wang et al., 2001; Kanayama et al., 2004). NF-κB essential modifier (NEMO) acts like a target of Lys63-linked polyubiquitination within the IKK complex and TRAF6-mediated polyubiquitination of NEMO was reported to be required for the optimal NF-κB activation by Toll-like receptor 4 (TLR4) (Abbott et al., 2007). In the IL-1 or LPS induced IL-1R1 or TLR4 signalling pathway, myeloid differentiation protein 88

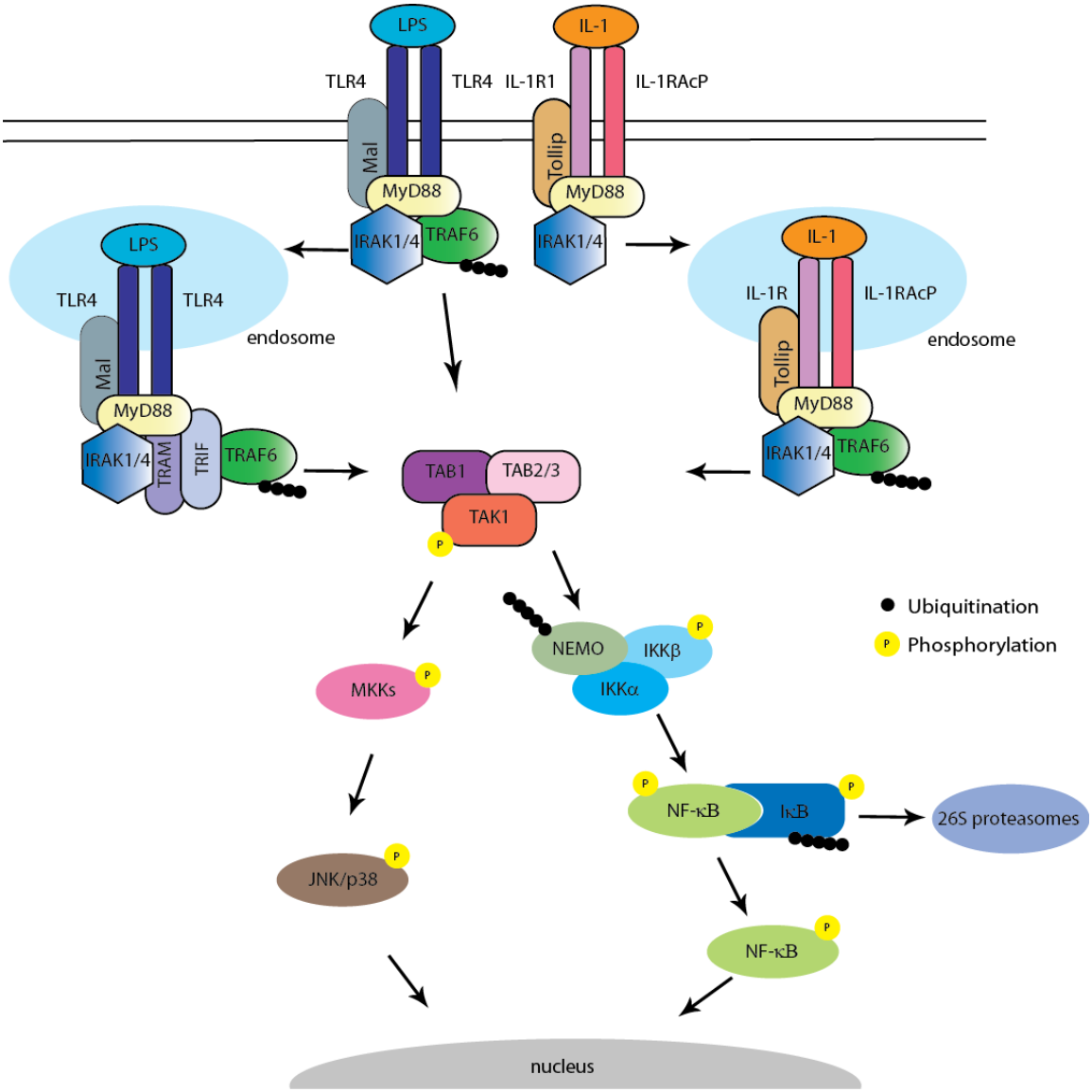


Figure 1.5 TRAF6 mediated IL-1R1/TLR4 signalling pathway. IL-1R/TLR4 stimulation leads to the formation of the complex with IL-1RAcP or TLR4 itself, allowing the recruitment of downstream adaptor including Mal or Tollip, MyD88 and IRAK1/4. Endocytosis of this complex is followed by recruitment of TRAF6 (require for TRIF and TRAM for TLR4). In combination with an E2 enzyme Ubc13, TRAF6 ubiquitinates and activates itself via Lys63-linked polyubiquitination chain. This autoubiquitination of TRAF6 leads to recruitment and activation of the downstream TAK1-TAB1-TAB2/3 complex. Both IKKβ and MKK family members are targets of TAK1 phosphorylation, and subsequently phosphorylate and activate IκB and JNK and p38 MAPKs. Activated inhibitor of NF-κB (IκB) is targeted by Lys48-linked polyubiquitination and is degraded by 26S proteasome, thus releasing NF-κB.

(MyD88) and IL-1 receptor associated kinase (IRAK) form complex with IL-1R1 or TLR4 after stimulation of IL-1 or LPS, and then TRAF6 is recruited as an adaptor protein in the endosome to the IL-1R1 or TLR4 complex, formation of which leads to the activation of NF-κB (Greenfeder et al., 1995b; Huang et al., 1997a; Wesche et al., 1997; Hull et al., 2002; Li et al., 2006b). Collectively, TRAF6 is involved in the activations of IKK, p38 and JNK induced by IL-1 and LPS. TRAF6 also plays an essential role in RANKL-dependent osteoclastogenesis by associating with RANK (Bai et al., 2005). Moreover, TRAF6 negatively regulates TNFα-induced NF-κB activation through its ubiquitin ligase activity (Funakoshi-Tago et al., 2009). TRAF6 also negatively regulates the Jak1-Erk pathway in IL-2 signalling by competing with Jak1 for IL-2R binding site (Motegi et al., 2011). More recently, TRAF6 has been shown to promote myogenic differentiation and muscle regeneration via the TAK1/p38 MAPK and Akt pathways suggesting its importance beyond inflammatory response (Xiao et al., 2012).

1.8 Role of TRAF6 and calcium signalling in osteoclastogenesis.

Association between TRAF6 and RANK is essential for RANKL-mediated NF-κB, c-Src kinase, Akt and PI3K activation (Galibert et al., 1998; Darnay et al., 1999; Wong et al., 1999). RANK is a critical signalling receptor modulating osteoclast differentiation, activation and survival (Lacey et al., 1998; Yasuda et al., 1998; Burgess et al., 1999; Lacey et al., 2000). Interaction of TRAF6 with RANK was shown to be required for the proper formation of cytoskeletal structures and functional bone resorption of

osteoclasts (Armstrong et al., 2002). Additionally, TRAF6 E3 ligase activity is required in regulating RANK signalling and osteoclast differentiation (Lamothe et al., 2007b). Deficiency of TRAF6 results in severe osteopetrosis *in vivo*, which was observed with defective interleukin-1 (IL-1), CD40 and lipopolysaccharide (LPS) signalling transductions (Lomaga et al., 1999b; Naito et al., 1999). In addition to RANKL-mediated osteoclast activation, TRAF6 is also reported to be essential for TNF-related apoptosis-induced ligand (TRAIL) induced osteoclast differentiation, suggesting a broader role of TRAF6 in regulating functions of osteoclast (Yen et al., 2012).

Calcium signalling also plays a critical role in the differentiation and functions of osteoclasts. RANKL signalling induces oscillatory changes in intracellular Ca^{2+} concentration (Takayanagi et al., 2002). Ca^{2+} oscillation results in dephosphorylation and activation of nuclear factor of activated T cell c1 (NFATc1), which translocates to the nucleus and induces osteoclast-specific gene transcription to trigger the differentiation of osteoclasts. Abolishment of RANKL-induced Ca^{2+} oscillation leads to impaired up-regulation of NFATc1 and osteoclastogenesis (Yang and Li, 2007), suggesting the importance of RANKL-induced Ca^{2+} oscillation in osteoclastogenesis. RANKL signalling triggers production of Inositol trisphosphate (IP3) through the activation of PLCγ, which leads to the release of Ca^{2+} from ER (Shinohara et al., 2008). Store-operated Ca^{2+} entry after Ca^{2+} release from ER is an important component of the Ca^{2+} oscillations, blocking of which results in abolished RANKL-

induced Ca^{2+} oscillations and bone resorption activity of osteoclasts (Mentaverri et al., 2003).

Skeletal defects have been observed in PS1 deficient mice (Shen et al., 1997; Mastrangelo et al., 2005) and these defects were attributed to disrupted γ-secretase cleavage of Notch. *In vitro* studies have shown that osteoclastogenesis can be regulated negatively by Notch1 signalling or positively by Notch2 signalling (Bai et al., 2008; Fukushima et al., 2008). However, Wnt signalling, which is negatively regulated by presenilin, is also required for osteoclast differentiation (Glass and Karsenty, 2006; Dobrowolski et al., 2012). Moreover, the APP Swedish mutation was suggested to regulate osteoclast differentiation in an age-dependent manner (Cui et al., 2011). Although presenilin itself was reported to function as passive ER Ca^{2+} leak channel, the role of this Ca^{2+} signalling event in osteoclastogenesis has not been discovered so far.

1.9 IL-1R1/TLR4 signalling and receptor post-translational modifications

Nine members of the IL-1 receptor (IL-1R) superfamily and thirteen members of the Toll-like receptor (TLR) superfamily have been discovered over the last decade. Besides the homologous cytoplasmic Toll/IL-1R resistance (TIR) domain, IL-1R superfamily members all contain extracellular Ig domains whereas TLR superfamily members contain Leucine-rich repeat domain in the N-terminus. Upon IL-1α/β binding, IL-1R1 associates with IL-1 receptor accessory protein (IL-1RAcP) (Greenfeder et al., 1995a; Huang et al., 1997b), leading to the recruitment of the TIR

domain containing adaptor MyD88, the serine/threonine kinase IL-1 receptor-associated kinase 1 (IRAK1), IRAK4 and the toll interacting protein (Tollip) (Wesche et al., 1997; Burns et al., 2000). IRAK1 contains three TRAF6 interaction consensus motifs in the C-terminal domain which enable the recruitment of TRAF6 (Ye et al., 2002). Activation of IRAK4 through its intramolecular autophosphorylation leads to the phosphorylation of IRAK1 and the full kinase activity (Kollewe et al., 2004; Cheng et al., 2007). IRAK1 and IRAK4 then dissociate from MyD88 and interact with TRAF6 while they still remain in the complex with IL-1R1 and IL-1RAcP (Deng et al., 2000; Brikos et al., 2007). TRAF6 is then activated by K63-linked autoubiquitination, dependent on the activity of E2 ubiquitin-conjugating complex Ubc13 and Uev1a (Deng et al., 2000). The IRAK/TRAF6 can then recruit TAK1 in a complex with TAB1 and TAB2 (or TAB3) and activation of TAK1 leads to the translocation of the complex to the cytosol (Qian et al., 2001; Jiang et al., 2002). Activated TAK1 then couples to the IKK complex containing NEMO and IKK where IKK is phosphorylated and activated by TAK1 which leads to the degradation of IκB and the consequent NF-κB activation (Wang et al., 2001). TAK1 also couples to the MKKs which leads to the activation of p38 and JNK. IRAK1 dissociated upon TAK1 activation remains in the membrane and undergoes K63-linked polyubiquitination mediated by Pellino (Butler et al., 2007; Schauvliege et al., 2007; Ordureau et al., 2008). It has been proposed that K63-linked polyubiquitination of IRAK1 recruits NEMO and may therefore provide an alternative pathway for NF-κB activation (Conze et al., 2008).

Signalling of TLR4 shares the general theme with IL-1R1 signalling but is more complex and involves a variety of adaptor proteins. Upon lipopolysaccharide (LPS) stimulation, TLR4 forms homodimers and recruits MyD88 through another TIR domain-containing adaptor, MyD88 adaptor like (Mal). Then the IRAK1/4 and TRAF6 are recruited which leads to the activation of the NF-κB pathway and MAPK pathways same as described above for IL-1R1 signalling. Additionally, the TLR4/Mal/MyD88 complex is also endocytosed to the early endosome where the TIR domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adapter molecule (TRAM) are recruited (Kagan et al., 2008). TRIF associates with TRAF6 to induce NF-κB activation and with receptor-interacting protein 1 (RIP1) to apoptosis (O'Neill and Bowie, 2007). This complex also activates TANK-binding kinase-1 and IKKε, leading to the dimerization and phosphorylation of the transcription factor interferon regulatory factor 3 (IRF3), which is required for activation of type I interferon promoters (Kawai and Akira, 2006).

Although many studies have been carried out to understand the functions IL-1R1/TLR4 signalling complexes and the exact role of each adaptor protein, very little is known about the post-translational modification of IL-1R1 and TLR4 themselves. Extracellular domain of IL-1R1 is glycosylated which is considered essential for its optimal binding to IL-1 (Mancilla et al., 1992). Similarly, glycosylation of TLR4 extracellular domain is essential for the LPS-induced activation of TLR4 signalling pathway as well as TLR4 plasma membrane expression (da Silva Correia and Ulevitch, 2002). Murine IL-1R1 is phosphorylated upon PMA treatment, but no consequent

effect was observed for this modification (Bird et al., 1991). IL-1R1 is also shown to be poly-ubiquitinated upon IL-1β stimulation and interact with two ubiquitin-binding proteins Tollip and Tom1, which modulates sorting of IL-1R1 in the late endosome to lysosome degradation (Brissoni et al., 2006). Besides of IL-1β, high glucose also triggers down regulation of IL-1R1 through lysosome degradation (Aveleira et al., 2010). The same study also observed translocation of IL-1R1 to the nucleus upon stimulation of IL-1β or high glucose. Previously, our group reported IL-1R1 as a substrate for γ-secretase-dependent regulated intramembrane proteolysis inhibition of which impairs its activation upon IL-1β stimulation (Elzinga et al., 2009a). An intracellular fragment was observed after γ-secretase cleavage of the C-terminus fragment, which is speculated to translocate to the nucleus. Furthermore, TRAF6 was shown to promote poly-ubiquitination of IL-1R1, increasing the cellular levels of full-length IL-1R1 protein as well as the C-terminus domain and the intracellular domain (Twomey et al., 2009).

1.10 TNFR1 signalling

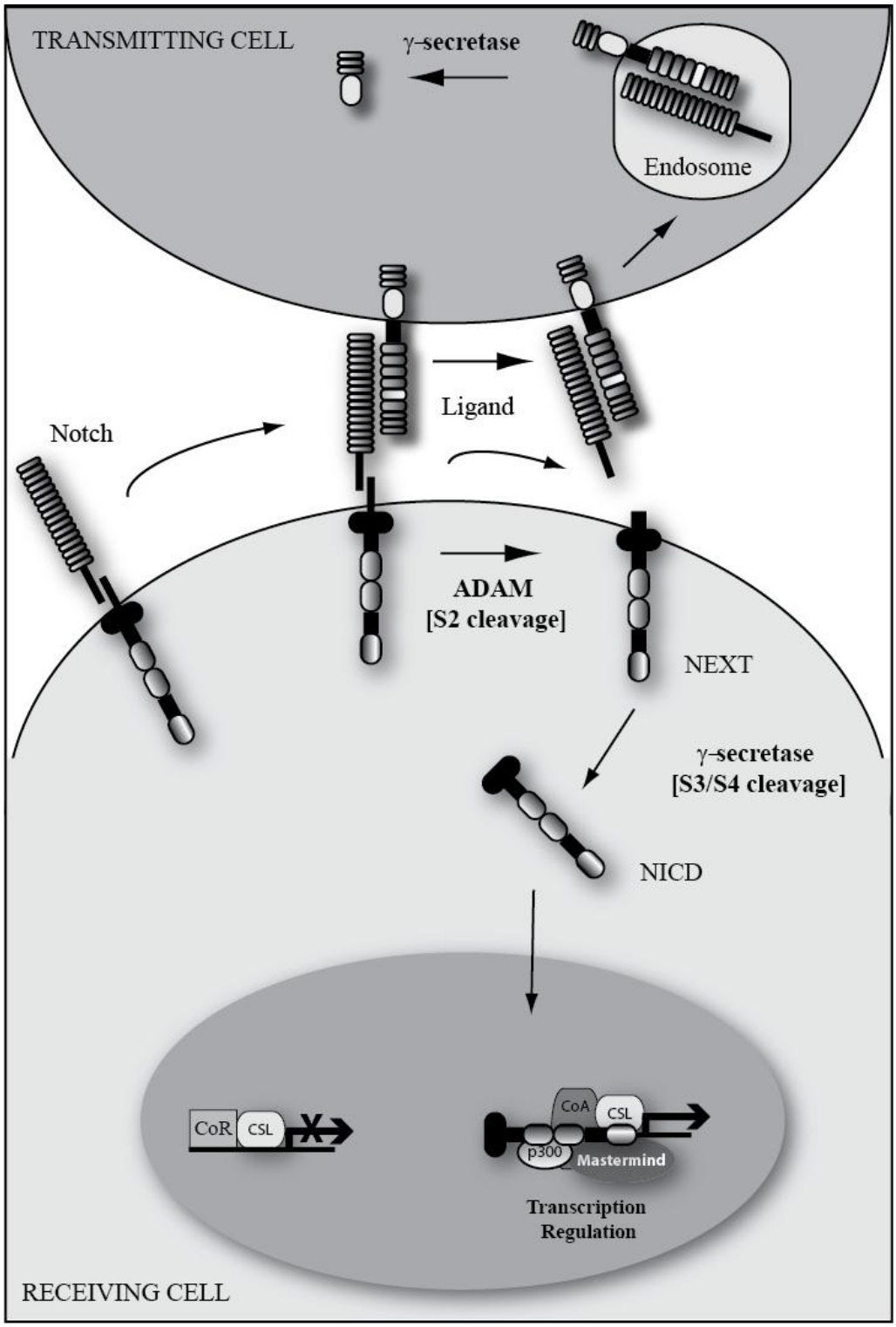
Not like IR-1R1 or TLR4, TNFR1 signalling is mediated by TRAF2 and TRAF5 (Au and Yeh, 2007). Knock-out of TRAF6 results in no defects in NF-κB activation in response to TNFα treatment (Lomaga et al., 1999a). However, TRAF6 is required for the signalling of two other TNFR superfamily members, CD40 and RANK (Lomaga et al., 1999a; Davies et al., 2005). Additionally, TRAF2 and TRAF6 are both required for TNFα-induced activation of mixed lineage kinase 3 (MLK3), which is a MAP3K

member (Korchnak et al., 2009). Stimulation of TNFα leads to the activation of TNFR1 and the recruitment of the signalling molecule TNFR associated death domain (TRADD) and the receptor interacting protein 1 (RIP1) (Hsu et al., 1996b; Hsu et al., 1996a). Activated complex is then relocated to lipid rafts, where TNFR1 and RIP1 become polyubiquitinated (Legler et al., 2003; Lee et al., 2004). RIP1 then recruits and activates TAK1 which leads to downstream activation of NF-κB. Another member of MAP3K, MEKK3, is also recruited to TNFR1 complex via RIP1 upon TNFα treatment (Yang et al., 2001; Blonska et al., 2004). MEKK3 then phosphorylates and activates IKKβ, providing a TAK1-independent pathway for NF-κB activation. TRAF5 has also been implicated in TNF-induced NF-κB activation, as depletion of TRAF2 only abolishes TNF-induced JNK activation whereas depletion of both TRAF2 and TRAF5 are required for abrogating NF-κB activation (Tada et al., 2001). In addition, activation of TNFR1 complex leads to internalisation and recruitment of Fas-associated death domain (FADD) and caspase-8 to induce apoptosis (Micheau and Tschopp, 2003). In contrast to the positive role of TRAF6 in IL1-R1/TLR4 signalling, TRAF2 and TRAF6 have been report to negatively regulate TNFR1 activity. Firstly, deficiency of TRAF2 results in hyperactivity of certain TNFR1 signals (Nguyen et al., 1999). Moreover, TNFα-induced IKK activation, IκBα degradation and transcriptional activation of NF-κB are all enhanced in TRAF6-deficient MEFs, suggesting that TRAF6 negatively regulates TNFα-induced NF-κB activation (Funakoshi-Tago et al., 2009).

1.11 Diversity of gamma-secretase substrates

Subsequent to the characterisation of γ-secretase-mediated proteolysis of APP and its association with the pathogenesis of AD, several independent groups have reported γ-secretase mediated proteolysis of more than 100 type-I membrane proteins, including Notch receptor **(Figure 1.5)** (Boulton et al., 2008; Lleo, 2008; McCarthy et al., 2009b; Haapasalo and Kovacs, 2011; Lleo and Saura, 2011). Although the absence of any immediately obvious or unifying biological function makes it difficult to predict whether or not a protein is a γ-secretase substrates, from the characterisation of known γ-secretase substrates there are emerging patterns that indicate the existence of some degree of regulation and unifying characteristics common to most substrate. Indeed all γ-secretase substrates are united in that all are (i) type-I integral membrane proteins (ii) have undergone ectodomain shedding as a result of proteolysis in their extracellular domains (iii) have a prerequisite for ectodomain shedding prior to γ-secretase cleavage of the remaining membrane-tethered C-terminal fragment. The lack of any significant substrate-specificity and ever increasing numbers of reported substrates has led to studies aimed at understanding the regulation of γ-secretase complexes and their proteolytic activities. The requirement for ectodomain shedding has led to the proposal that some degree of regulation must surround subsequent γ-secretase cleavage, which is supported by the observed ligand-induced ectodomain shedding and γ-secretase cleavage of many substrates. Likewise, posttranslational modification of certain γ-secretase substrates has been shown to regulate substrate cleavage, where

Figure 1.5 Regulated Intramembrane proteolysis and Notch signalling. Cell surface heterodimeric Notch receptor is activated by binding to ligand presented by a neighbouring transmitting (signal) cell, and activates a signalling cascade through sequential proteolytic cleavage events. Ligand binding is proposed to induce a conformational change in the ligand-bound receptor, enabling Notch extracellular domain cleavage at site 2 (S2) by a disintigrin and metalloproteinase (ADAM) protease, release of Notch extracellular domain and generation of the membrane-anchored Notch extracellular truncation (NEXT) fragment. Subsequently, NEXT is progressively cleaved within the transmembrane domain by γ-secretase at site 3 (S3) and site 4 (S4) cleavage sites to generate the cytosolic Notch intracellular domain (NICD). Notch cleavage by γ -secretase can occur at the plasma membrane or endosomal compartments producing NICD fragments with different stability. NICD then enters the nucleus where it interacts with the DNA-binding protein CSL (CBF1/RBPjκ/Su (H)/Lag-1), releases co-repressors (CoR), and enables recruitment of transcriptional co-activators Mastermind and p300. These interactions convert CSL from a transcriptional repressor to an activator. Notch ligands (Delta and Jagged) are also γ-secretase substrates, though the physiological consequence remains to be determined.


TRANSMITTING CELL
γ-secretase
Endosome
Ligand
Notch
ADAM
[S2 cleavage]
NEXT
γ-secretase
[S3/S4 cleavage]
NICD
CoR
CSL
CoA
CSL
p300
Mastermind
Transcription
Regulation
RECEIVING CELL

ubiquitination of human Notch-CTD (Gupta-Rossi et al., 2004a) and Interleukin-1 receptor type 1 (IL-1R1) (Twomey et al., 2009) and palmytolation of the p75NTR (Underwood et al., 2008) are a prerequisite for γ-secretase cleavage. However, recent data suggest that posttranslational modification may not be a general requirement for cleavage of all γ-secretase substrates (McCarthy et al., 2009b). One further and important factor that requires further investigation arises from studies proposing that γ-secretase activity and cleavage of certain substrates may be determined by their spatiotemporal location (Hass et al., 2008), prompting more rigorous *in vivo* studies.

Despite the fact that all reported substrates have little or no sequence homology around their cleavage sites mutagenesis studies on APP, Notch and other substrates have identified certain hydrophobic residues in the transmembrane and intracellular juxtamembrane domains that determine γ-secretase cleavage efficiency and importantly, substrate-specificity (Schroeter et al., 1998; Weidemann et al., 2002). These reports have led to the proposed hypothesis that γ-secretase requires subtle sequence or structural characteristics, which enable substrate recognition and cleavage. Likewise, certain PS1 variants associated with FAD have been shown to differentially affect APP proteolysis at the ε- and γ-cleavage sites (Tolia et al., 2006; Tolia et al., 2008). Indeed, site-directed mutagenesis studies have reported that substitution of a phenylanine at position x of the GxGD aspartyl protease active site motif of presenilin results in preferential cleavage of APP over Notch (Yamasaki et al., 2006), while a single substitution (I437C) in TMD9 renders γ-secretase inactive

towards cleavage of Notch, but not APP (Tolia et al., 2008). Based on the diversity of reported γ-secretase substrates, there are a number of proposed functional consequences of regulated intramembrane proteolysis. Firstly, it is proposed that following receptor-ectodomain shedding, γ-secretase may be involved in the proteolytic removal of membrane-anchored protein fragments from the membrane, acting as the proteasome of the membrane (Kopan and Ilagan, 2004). Secondly, it has been demonstrated for certain specific substrates, that the γ-secretase generated intracellular domain (ICD) has a central role in mediating ligand-initiated nuclear signalling events whereby the ICD can translocate to the nucleus and regulate gene transcription (Pardossi-Piquard et al., 2005; Hebert et al., 2006; Sardi et al., 2006; Wolfe, 2008a; Carpenter and Red Brewer, 2009; Maetzel et al., 2009). Thirdly, γ-secretase cleavage of substrates may function to initiate a novel receptor-independent function whereby the soluble ICD can translocate to another intracellular compartment to initiate a novel biological function (Boulton et al., 2008; Hass et al., 2008). Finally, γ-secretase cleavage may also function to terminate a transmembrane receptor-mediated signalling event (Jung et al., 2003). In summary, although increasing numbers of γ-secretase substrates are being identified and attributed *in vitro* biological functions (Hass et al., 2008; McCarthy et al., 2009b), no unifying regulatory mechanism controlling γ-secretase activity or common *in vivo* biological consequence of γ-secretase cleavage has emerged. To date there are only three γ-secretase substrates wherein an *in vivo* physiological signalling function has been convincingly attributed to γ-secretase cleavage, further highlighting the need

for more rigorous *in vivo* studies to delineate the physiological importance of γ-secretase-mediated cleavage of each reported substrate (Louvi and Artavanis-Tsakonas, 2006; Sardi et al., 2006; Wang et al., 2008).

Having given the description of covalent and proteolytic posttranslational modifications of presenilins, the existence of six subtypes of γ-secretase complex, a diversity of γ-secretase interacting partners and substrates and the complexity of the signalling pathways of IL-1R1, TLR4 and TNFR1, it is self-evident that a comprehensive understanding of the regulation and mechanism of γ-secretase complexes and presenilins as well as of the γ-secretase substrates (IL-1R1/TLR4/TNFR1) will be requisite for the development of cell biology and therapeutic strategies for AD and immune disorders.

1.12 Research objective

The aim of this research project was to characterise and determine the importance of the interaction between presenilins and TRAF6 and the regulation of IL-1R1 by TRAF6. TRAF2 and TRAF6 are E3 ubiquitin ligases that specifically interact with the presenilin proteins, suggesting that this interaction serves as a novel means of regulating presenilin functions and γ-secretase activity. Additionally, TRAF2, TRAF5 and TRAF6 have been reported to form signalling complexes with TNFR1 and IL-1R1/TLR4 respectively and to be essential for the signalling transduction. However, the roles of the TRAF regulation on the receptors themselves have not been widely studied. This project employed *in vitro* cell-based experimental approaches, recombinant DNA technologies, co-immunoprecipitation and immunobiology to determine the importance of the interactions between presenilins and TRAFs and their effects on presenilin and γ-secretase activity. Moreover, regulation of IL-1R1 by TRAF6 has been further explored, revealing the novel role of TRAF6 other than adaptor protein. This study yields new insights into TRAF6-mediated presenilin and IL-1R1 functions and contributes to our understanding of the regulation and complexity of presenilin biology and IL-1R1 signalling.

Chapter 2:

MATERIALS AND METHODS

2.1 Materials

2.1.1 General chemicals and reagents

All salts, reagents and Anti-Flag Agarose Beads were purchased from Sigma-Aldrich (Dublin, Ireland) unless otherwise stated. Protein G-sepharose beads and DNA ladder were procured from Invitrogen (Paisley, Scotland). Prestained molecular weight protein markers were purchased from BioRad Laboratories (GmbH, Munich, Germany). Bicinoic acid (BCA) protein concentration reagents were purchased from Pierce Biotechnology (Rockford, Illinois) through Medical Supply Company (MSC) Ltd. (Dublin, Ireland). Nitrocellulose membrane and Western blotting filter paper were purchased from Schleicher and Schuell (GambH, Dassel, Germany). Complete protease inhibitor tablets were purchased from Roche (Boehringer-Mannheim, Indianapolis, USA).

2.1.2 Molecular biology reagents

All restriction enzymes were purchased from New England Biolabs through ISIS (Co. Wicklow, Ireland). Maxiprep kits was purchased from Qiagen Ltd. (West Sussex, UK).

2.1.3 Plasmid sources

HA-tagged ubiquitin, HA-tagged all-lysine-mutated ubiquitin (ubiquitin K0) and HA-tagged K63R ubiquitin were provided by Dr Ruaidhri Carmody (University of Glasgow, UK). PDLIM2 and β-TRCP were gifts from Dr Rosemary O'Connor (University College Cork, Ireland). PS1 NTF, PS1 CTF and APP CT100 were gifts from Scios Incorporation. All TRAF constructs, TNFR1 and IL1-R1 were gifts from Genentech Inc. The rest of the

constructs listed below were generated previously in our lab. The following plasmids were used:

Construct	Vector	Source	Epitope Tag
APP SW	pcDNA3	Scios Inc.	untagged
APP CT100	pcDNA3	Scios Inc.	untagged
Empty Vector	pcDNA3	Invitrogen	untagged
IL-1R1 WT	pcDNA3	Genentech Inc.	untagged
PS1 WT	pcDNA3	(Powell et al., 2009)	untagged
PS1 D257A/D385A	pcDNA3	(Elzinga et al., 2009b)	untagged
PS1 L271A	pcDNA3	created in lab	untagged
PS1 F283A	pcDNA3	created in lab	untagged
PS1 V309A	pcDNA3	created in lab	untagged
PS1 S310A	pcDNA3	created in lab	untagged
PS1ΔCUE	pcDNA3	created in lab	untagged
PS2 WT	pcDNA3	(Elzinga et al., 2009b)	untagged
PS2ΔCUE	pcDNA3	created in lab	untagged
PS1 WT	Nse	Scios Inc.	untagged
PS1 NTF	Nse	Scios Inc.	untagged
PS1 CTF	Nse	Scios Inc.	untagged
PDLIM2	Pcmv	(Loughran et al., 2005)	Myc
TRAF2	pRK 5	Genentech Inc.	FLAG

TRAF3	pRK 5	Genentech Inc.	FLAG
TRAF4	pRK 5	Genentech Inc.	FLAG
TRAF5	pRK 5	Genentech Inc.	FLAG
TRAF6	pRK 5	Genentech Inc.	FLAG
TRAF6 DN	pRK 5	Genentech Inc.	FLAG
TRAF2 DN	pRK 5	Genentech Inc.	FLAG
TRAF6 C70A	pRK 5	(Twomey et al., 2009)	FLAG
TRAF6 K124A	pRK 5	(Twomey et al., 2009)	FLAG
β-TRCP	pAS1B	Dr. Rosemary O'Connor	HA
TLR4	pcDNA3	Addgene	YFP
TNFR1	pcDNA3	Genentech Inc.	untagged
Ubiquitin WT	Unknown	(Carmody et al., 2007)	HA
Ubiquitin K63R	Unknown	(Carmody et al., 2007)	HA
Ubiquitin K0	Unknown	(Carmody et al., 2007)	HA

The PS1, PS2 and IL-1R1 mutants which were created in our lab used human *PSEN1* (NCBI NM_000021.3), human *PSEN2* (NCBI NM_000447.2) and human IL-1R1 (NCBI NM_000877.2) sequences as templates respectively. Names of the mutants and the corresponding primers are listed below.

PS1ΔCUE:

PS1 forward 5' CGGGGTACCGCCATGACAGAGTTACCTGCACCGTTGTCC 3' Kpn1

PS1ΔCUE reverse 5' TTCTGCATTATAAGCCACTAAATCATATACTGA 3'

PS1ΔCUE forward 5’ GATTTAGTGGCTTATAATGCAGAAAGCACAGAA 3’

PS1 reverse 5’ CCGGAATTCCTAGATATAAAATTGATGGAATGCTAATTG 3’ EcoR1

PS1ΔCUE:

PS2ΔCUE reverse 5’ TTCCATCTCCGGAGCCACGAGATCATACACAGA 3’

PS2ΔCUE forward 5’ GATCTCGTGGCTCCGGAGATGGAAGAAGACTCC 3’

PS2 forward 5’ GCCGGGATCCGCCATGCTCACATTCATGGCCTCTG 3’ BamH1

PS2 reverse 5’ GCCGGAATTCTCAGATGTAGAGCTGATGGGAG 3’ EcoR1

PS1 L271A forward 5' GAGAGAAATGAAACCGCTTTTCCAGCTCTCATTTAC 3'

PS1 L271A reverse 5' GTAAATGAGAGCTGGAAAAGCCGTTTCATTTCTCTC 3'

PS1 F283A forward 5’ GAGAAATGAAACGCTTGCTCCAGCTCTCATTTACTCC 3’

PS1 F283A reverse 5’ GGAGTAAATGAGAGCTGGAGCAAGCGT TTCATTTCTC 3’

PS1 V309A forward 5’ GGGAAGCTCAAAGGAGAGCATCCAAAAATTCCAAG 3’

PS1 V309A reverse 5’ CTTGGAATTTTTGGATGCTCTCCTTTGAGCTTCCG 3’

PS1 S310A forward 5’ CGGAAGCTCAAAGGAGAGTAGCCAAAAATTCCAAG 3’

PS1 S310A reverse 5’ CTTGGAATTTTTGGCTACTCTCCTTTGAGCTTCCG 3’

PS1 K76RK80R forward 5’ ACATTGAGATATGGCGCCAGGCATGTG 3’

PS1 K76RK80R reverse 5’ CACATGCCTGGCGCCATATCTCAATGT 3’

PS1K109R forward 5’ TACCCGGAGGGATGGGC 3’

PS1K109R reverse 5’ GCCCATCCCTCCGGGTA 3’

PS1 K155RK160R forward 5’ TTCTGTATAGATACAGGTGCTATAGGGTCATCCATG 3’

PS1 K155RK160R reverse 5’ CATGGATGACCCTATAGCACCTGTATCTATACAGAA 3’

PS1 K187R forward 5’ GTGTTTAGAACCTATAACG 3’

PS1 K187R reverse 5’ CGTTATAGGTTCTAAACAC 3’

PS1 K216R forward 5’ TCACTGGAGAGGTCCACTTCG 3’

PS1 K216R reverse 5’CGAAGTGGACCTCTCCAGTGA 3’

PS1 K265R forward 5’ TGTCCGAGAGGTCCACTTCG 3’

PS1 K265R reverse 5’ CGAAGTGGACCTCTCGGACA 3’

PS1 K311RK314R forward 5’ GTATCCAGAAATTCCAGGTATAATGC 3’

PS1 K311RK314R reverse 5 ’GCATTATACCTGGAATTTCTGGATAC 3’

PS1 K380R forward 5’ GGGGAGTAAGACTTGGATTGG 3’

PS1 K380R reverse 5’ CCAATCCAAGTCTTACTCCCC 3’

PS1 K429RK430R forward 5’ CATTTTCAGGAGAGCATT 3’

PS1 K429RK430R reverse 5’ AATGCTCTCCTGAAAATG 3’

IL1-R1 1-540 forward CGGGGTACCGCCATGAAAGTGTTACTCAGACTTATTTG Kpn1

IL1-R1 1-540 reverse CCGGAATTCCTACCTGACATTCTTCCAGAACC EcoR1

IL1-R1 1-500 forward CGGGGTACCGCCATGAAAGTGTTACTCAGACTTATTTG Kpn1

IL1-R1 1-500 reverse CCGGAATTCCTATGGCATTTTCTCATAGTCTTGG EcoR1

IL1-R1 1-440 forward CGGGGTACCGCCATGAAAGTGTTACTCAGACTTATTTG Kpn1

IL1-R1 1-440 reverse CCGGAATTCCTAAATGACCTCAACAATGTCTTCCCC EcoR1

IL1-R1 1-390 forward CGGGGTACCGCCATGAAAGTGTTACTCAGACTTATTTG Kpn1

IL1-R1 1-390 reverse CCGGAATTCCTACAGTATATATGCGTCATAGGTCTTTCC EcoR1

IL1-R1 1-356 forward CGGGGTACCGCCATGAAAGTGTTACTCAGACTTATTTG Kpn1

IL1-R1 1-356 reverse CCGGAATTCCTAATAGATGAAAACAGAACACAC EcoR1

IL1R1 K445446R forward 5’ GAAAACGTAAGGAGAAGCAGAAGAC 3’

IL1R1 K445446R reverse 5’ GTCTTCTGCTTCTCCTTACGTTTTC 3’

IL1R1 K504507R forward 5’ GAATCGATTAGATTCATTAGGCAGAAACATG 3’

IL1R1 K504507R reverse 5’ CATGTTTCTGCCTAATGAATCTAATCGATTC 3’

IL1R1 K527532R forward 5’ CAGTCTGCAAGGACAAGGTTCTGGAGGAATGTCAGG 3’

IL1R1 K527532R reverse 5’ CCTGACATTCCTCCAGAACCTTGTCCTTGCAGACTG 3’

IL1R1 K548R forward 5’ CCTTCATCTAGACACCAGTTAC 3’

IL1R1 K548R reverse 5’ GTAACTGGTGTCTAGATGAAGG 3’

IL1R1 K378383R forward 5’ CCAATAAGAGCTTCAGATGGAAGGACCTATG 3’

IL1R1 K378383R reverse 5’ CATAGGTCCTTCCATCTGAAGCTCTTATTGG 3’

IL1R1 K393R forward 5’ GCATATATACTGTATCCAAGGACTGTTGGGGAAGG 3’

IL1R1 K393R reverse 5’ CCTTCCCCAACAGTCCTTGGATACAGTATATATGC 3’

IL1R1 K409R forward 5’ GTGATATTTTTGTGTTTAGAGTCTTGCCTGAGG 3’

IL1R1 K409R reverse 5’ CCTCAGGCAAGACTCTAAACACAAAAATATCAC 3’

IL1R1 K417422R forward 5’ CTTGGAAAGACAGTGTGGATATAGGCTGTTC 3’

IL1R1 K417422R reverse 5’ GAACAGCCTATATCCACACTGTCTTTCCAAG 3’

IL1R1 K357360R forward 5’ CTGTTTTCATCTATAGAATCTTCAGGATTGACATTGTG 3’

IL1R1 K357360R reverse 5’ CACAATGTCAATCCTGAAGATTCTATAGATGAAAACAG 3’

IL1R1 K360R forward 5’ CATCTATAAAATCTTCAGGATTGACATTGTG 3’

IL1R1 K360R reverse 5’ CACAATGTCAATCCTGAAGATTTTATAGATG 3’

IL1R1 K378R forward 5’ CCAATAAGAGCTTCAGATGGAAAGACCTATG 3’

IL1R1 K378R reverse 5’ CATAGGTCTTTCCATCTGAAGCTCTTATTGG 3’

PS1 K76/80R, PS1 K109R, PS1 K155/160R, PS1 K187R, PS1 K216R, PS1 K265R, PS1 K311/314R, PS1 K380R, PS1 K429/430R, IL-1R1 K357/360R, IL-1R1 K378/383R, IL-1R1 K393R, IL-1R1 K409R, IL-1R1 K417/422R, IL-1R1 K445/446R, IL-1R1 K504/507R, IL-1R1 K527/532R, IL-1R1 K548R, IL-1R1 K360/378R, IL-1R1 K356/360/378R, IL-1R1 K360/378/383R, IL-1R1 K357/360/378/383R, IL-1R1 K360/378/383/527/532R are all made by site-directed mutagenesis as described below. PS1ΔCUE, PS2ΔCUE, IL-1R1 1-535, IL-1R1 1-500, IL-1R1 1-440, IL-1R1 1-390, IL-1R1 1-356 are generated by PCR and DNA ligation in our lab.

2.1.4 Antibodies

All antibodies were obtained from commercial sources: Rat anti-human PS1-NTF and anti-human PS1-CTF were purchased from Chemicon (Hamphshire, UK); anti-HA were purchased from Covance (Berkely, California, USA); anti-Nicastrin antibody was purchased from BD Biosciences; anti-β-actin, anti-myc, anti-FLAG, anti-APP CTF and anti-β-Tubulin were purchased from Sigma-Aldrich (Dublin, Ireland); anti-Ubiquitin (P4D1), anti-p-JNK, anti-JNK1, anti-TRAF6, anti-Aph1, anti-PEN2, anti-IL-1R1 C20, anti-IL-1R1 N20 and TLR4 antibodies were purchased from Santa-Cruz Biotechnology (California, USA); polyclonal rabbit anti-human PS2 CTF and anti-TNFR1 antibodies were obtained from Cell Signaling Technology (Danvers, Massachusetts, USA). Infrared secondary antibodies IRDye® 680 Goat Anti-Rabbit IgG and IRDye® 800CW

Goat Anti-Mouse IgG were purchased from Licor Biosciences (Cambridge, UK). IRDye® 800CW Goat anti-Mouse lgG F(c) was purchased from Rockland through tebu-bio (Dublin, Ireland).

2.1.5 Cell lines

Human Embryonic Kidney 293T (HEK293T) cells from lab stocks (originally purchased from ATCC) were cultured in cells were grown in Dulbecco's modified Eagle's medium DMEM-21 containing 10% (v/v) fetal bovine serum, 1% (v/v) L-glutamine, and antibiotics (50units/ml penicillin and 50µg/ml streptomycin). Fibroblasts derived from PS1/PS2-deficient mouse embryos were isolated as previously described (De Strooper et al., 1998). Fibroblast cell lines were generated by transformation of primary cultures with large T antigen (De Strooper et al., 1998). All murine embryonic fibroblast (MEF) cell lines were grown in DMEM-21, high glucose, 10% (v/v) FBS, 1% (v/v) glutamine, 1% (v/v) non-essential amino acids, 1% (v/v) sodium pyruvate, and penicillin/streptomycin with 5% (v/v) CO_2. Cells were maintained in a humidified 37°C incubator with 5% CO_2. All reagents were purchased from Sigma-Aldrich (Dublin, Ireland).

2.1.6 Bacterial strains and media used

Lab stocks of the E. coli DH5α strain were grown in pre-made Luria-Bertani (LB) broth and LB agar which were purchased from Sigma-Aldrich (Dublin, Ireland).

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Computer software used in Bioinformatical analysis

DNA sequence analysis and alignments were performed using Vector NTI.

2.2.1.2 Competent cell preparation and transformation

DH5α were streaked for single colonies and grown on an LB plate overnight at 37°C. A single colony was picked and grown in 5ml of LB medium overnight at 37°C. 3ml of overnight grown DH5α cells were inoculated into 100-200ml of LB solution and grown for 2-3 hours. Once cell density reached OD_{595} 0.6-1.0, cells were harvested by centrifugation of cells at 3000x g for 15min at 4°C. The supernatant was discarded and the pellet resuspended in pre-cooled 0.1 M $MgS0_4$ at one third the volume of the bacterial culture volume. Cells were then harvested as above and the pellet resuspended in 0.1 M $CaCl_2$ containing 15% (v/v) of glycerol with 1/25 of bacterial culture volume. Aliquots were put in pre-cooled tubes, frozen on dry ice and then stored at -80°C.

2.2.1.3 Transformation of DH5α competent cells

Competent cells were thawed on ice for 15-20 minutes. Approximately 1ng of a plasmid DNA or 2-18 µl of a ligation mixture was mixed with 100 µl of competent cells and incubated on ice for 15-45 minutes. Cells were heat-shocked at 42°C for 90 seconds and immediately cooled on ice. 900 µl of LB broth was added and incubated at 37°C shaking for 50-90 minutes. 20-200 µl of the cell suspension was spread on a

pre-warmed LB agar plate containing an appropriate antibiotic (50µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37°C overnight.

2.2.1.4 Site-directed mutagenesis

Primers used for site-directed mutagenesis were designed using Vector NTI 11. KOD Hot Start Polymerase kit (#71086) from Millipore was used for the PCR reactions. The standard reaction setup is shown as below.

Component	Volume	Final Concentration
10X Buffer for KOD Hot Start DNA Polymerase	5 µl	1X
25 mM $MgSO_4$	3 µl	1.5 mM
dNTPs (2 mM each)	5 µl	0.2 mM (each)
PCR Grade Water	32 µl	
Sense (5') Primer (10 µM)	1.5 µl	0.3 µM
Anti-Sense (3') Primer (10 µM)	1.5 µl	0.3 µM
Template DNA 10 ng/µl	1 µl	0.2 ng/µl
KOD Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02U/µl
Total reaction volume	**50 µl**	

The PCR programme setup is listed below.

1. Polymerase activation	95°C for 2 min
2. Denature	95°C for 20 s
3. Annealing	Lowest Primer Tm°C for 10 s
4. Extension	70°C for 25s/kb
Repeat 2-4	20 cycles
5. Final extension	70°C for 5 min

The PCR products were purified using Qiagen PCR purification kit (#28106). Purified PCR products were digested by DpnI restriction enzyme as described below. Then the PCR products were transformed in DH5α competent cells for further plasmid preparation and sequencing analysis.

2.2.1.5 Plasmid DNA restriction digest and DNA gel electrophoresis

Following NEB guildlines (http://www.neb.com/nebecomm/default.asp) add the following to an Eppendorf tube: 500ng plasmid DNA, 2µl 10x NEBuffer, 0.5µl 100xBSA (if necessary), 0.5µl Restriction Enzyme (of one or more) and add dH_2O to make the final volume 20µl. Mix and incubate at 37°C for 1-2 hours. Samples were then mixed with 5µl DNA running dye (10mM EDTA, 0.25% Bromophenol Blue, 50% Glycerol). Samples were loaded onto a 1% Agarose gel containing 0.5µg/ml of Ethidium Bromide so that DNA can be visualised and DNA was separated with an applied potential difference of 100V for about 1 hour. Gel was observed by illuminating on a 302 nm UV transilluminator. Commercially obtained DNA ladders were used as molecular weight markers.

2.2.2 Cell Biology

2.2.2.1 Calcium phosphate transfection of HEK293T cells

Transfection was carried out on subconfluent human embryonic kidney (HEK293T) cell cultures using the calcium phosphate precipitation method. For a 10cm dish with 10mls of culture media, 2.5 µg of each plasmid was added to 62µl 2M $CaCl_2$ with up

to 438 µl of sterile water. This solution was added drop-wise over the course of two minutes to 500µl Hanks buffered salt solution (HBSS) and the resulting cloudy mixture was allowed to stand a further one minute. The 1ml precipitate solution was then added to the cell culture. Essentially the same procedure was performed for each well of a 6-well plate except 200 µl of the precipitate solution containing 1 µg of total DNA was added per well. In all instances the total amount of DNA in each culture was kept constant by addition of empty vector pcDNA3 (Invitrogen). Media was changed 8-16 hours post-transfection and cultures were harvested 24-48 hours post-transfection.

2.2.2.2 Transfection of murine embryonic fibroblasts (MEF's)

Transfection was carried out on subconfluent MEF cell cultures using the TurboFect™ in vitro Transfection Reagent from BioRad Laboratories (GmbH, Munich, Germany). Prior to transfection, reagent was thawed and cell-culture media was replaced. Two hours later 2µg of plasmid DNA was diluted in 200µl serum free DMEM medium. 4µl of TurboFect™ was added to the DNA solution and mixed thoroughly. This solution was incubated for 20 minutes at room temperature. Then the combined solution was added drop-wise to 10 cm plates.

2.2.2.3 Preparation of cellular protein extracts

HEK293T cell cultures were washed with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Nonidet P-40, 1 mM sodium orthovanadate and protease inhibitor mixture

(Complete™, Roche Molecular Biochemicals). Cells were lysed on ice for 30 minutes, spun at 21,920 x g at 4°C for 10 minutes and supernatants were collected. Lysates were normalised using the bicinchonic acid (BCA) method by Pierce.

MEF cell cultures were transferred on ice to a 4°C cold-room (this is to prevent degradation of endogenous proteins by proteases) and cell cultures were washed with ice-cold phosphate-buffered saline (PBS). Removing any excess PBS, next a solution of RIPA buffer (50mM Tris-Cl, pH 7.4, 0.15m NaCl, 1% sodium deoxycholate, 1% Triton X-100, 10mM EDTA, 0.1% SDS) containing 1x complete protease inhibitor was added (for 100 mm cell-culture dishes 1 ml was added). Cells were incubated with RIPA buffer for at least half an hour and then cells were removed by cell scraper. Cells were then transferred to eppendorf and were pushed through a syringe and needle 10 times to be broken up into viscous lysate. Lysate was finally spun down at 21,920 x g at 4°C for 25 minutes and supernatants were collected. Lysate was normalised to ensure an equal amount of protein loaded to each sample using the bicinchonic acid (BCA) method by Pierce.

2.2.2.4 Immunoprecipitation of proteins from cellular extracts

Equivalent concentrations of lysates (for exogenously expressed proteins 200-500 µg of protein lysate and for endogenously expressed proteins 1000 µg of protein lysate) were pre-cleared for 1 hour at 4°C with 25 µl Protein-G sepharose beads. Pre-cleared lysates were immunoprecipitated for 2 hours at 4°C with 2-5 µg of the indicated antibody followed by incubation with 25 µl Protein-G sepharose beads overnight. Immunoprecipitates were then washed three times in 500 mM NaCl lysis buffer

followed by two washes in 150 mM NaCl lysis buffer. Samples were resolved by 10 % or 12 % SDS-PAGE, transferred to nitrocellulose membrane and visualised as outlined below.

2.2.2.5 Immunoprecipitation of proteins from cellular extracts for ubiquitination analysis

Cell cultures were washed twice in ice-cold PBS and detached from plates by gentle scraping in 1 ml PBS-EDTA (0.5 mM). The resulting suspensions were transferred into 1.5 ml eppendorf and a sample removed for lysis and Western blot analysis as earlier described. Cells were harvested by spinning at 400 x g for 5 minutes at 4°C. Pellets were resuspended in 250 µl 1% (w/v) Sodium Dodecyl Sulphate (SDS) containing 15mM N-ethylmaleimide (NEM) and protease inhibitors and boiled for 5 minutes on a heating block. Following cooling on ice, an equal volume of ice-cold covalent buffer (50mM Tris (pH 8.0), 150mM NaCl, 1 % Triton, 0.5 % sodium deoxycholate, 15 mM NEM and protease inhibitors) was added, lysate was mixed and spun at top speed at 4°C for 20 minutes. The pellet was removed and lysates quantified using the BCA method as previously described. Samples were then subjected to immunoprecipitation with indicated antibodies as described previously, except that Protein-G sepharose beads were washed three times in covalent buffer.

2.2.2.6 Western blotting

Equivalent concentrations of lysates were denatured by addition of SDS loading buffer and boiling for 5 minutes on a heating block. However when blotting for PS1

or PS2, the boiling step was omitted as boiling PS1 or PS2 disrupts the epitope-antibody recognition site (unpublished results J.V McCarthy). Similarly, washed Protein G beads were boiled in SDS loading buffer and boiled for 5 minutes on a heating block. Samples were resolved on 10 % (w/v) or 12 % (w/v) SDS-PAGE gels, transferred to nitrocellulose membrane (Schleicher and Schuell Bioscience). Following transfer, membranes were blocked for 1 hour at room temperature in 5 % (w/v) Marvel milk/PBS-Tween 0.1 % (v/v). Primary antibodies were diluted as outlined below in 5 % (w/v) Marvel milk/PBS-Tween 0.1 % (v/v) and incubated on the shaking membranes 4°C overnight.

Antibody Name	**Company Code**	**Working Dilution**
Anti-PS1-CTF	MAB5232	1:1000
Anti-PS1-NTF	MAB1563	1:1000
Anti-PS2-CTF	#2192	1:1000
Anti-HA	MMS-101R-200	1:3000
Anti-β-actin	A5316	1:3000
Anti-FLAG	F3165	1:3000
Anti-Tubulin	T4026	1:3000
Anti-APPCTF	A8717	1:4000

Anti-p75	G3231	1:1000
Anti-IL-1R1 C20	SC-687	1:500
Anti-Nicastrin	N16420-150	1:4000
Anti-Myc	M5546	1:3000
Anti-TNFR1	#3736S	1:1000
Anti-p-JNK	SC-6254	1:200
Anti-JNK1	SC-571	1:200
Anti-TRAF6	SC-8409	1:200
Anti-Aph1	SC-30240	1:200
Anti-PEN2	SC-32946	1:200
Anti-ubiquitin	SC-8017	1:200
Anti-IL-1R1 N20	SC-688	1:200
Anti-TLR4	SC-10741	1:200

Membranes were washed three times in PBS-Tween or TBS-Tween for 10 minutes each and incubated in diluted secondary antibody for 1 hour at room temperature. 1:10000 dilution in 1 % (w/v) Marvel/PBS-T/TBS-T was used for Licor antibodies. Following washing, proteins were detected with the Licor Odyssey Infrared Imaging System for Licor antibodies.

2.2.2.7 ELISA for Aβ40 and Aβ42

HEK293T cells were transfected by calcium phosphate precipitation with indicated constructs. Thirty-six hours after transfection, cell culture medium was collected for ELISA analysis and cells were subjected to Western blotting. ELISA kits for Aβ40 and Aβ42 were purchased from Invitrogen (#KHB3481 and #KHB3441). ELISA analyses were carried out according to the manufacturer's protocol.

2.2.2.8 Reverse transcription PCR and Real-time PCR

Thirty-six hours post transfection, HEK293T cells were subjected to total RNA extraction using High Pure RNA Isolation Kit from Roche (#11828665001). 5 µg total RNA was then applied in reverse transcription PCR using Super Script III reverse transcriptase from Invitrogen (#18080-044) following the manufacturer's protocol. Then the Real-time PCR was set up using LightCycler 480 SYBR Green I Master kit from Roche (#04707516001) following the manufacturer's protocol. Reactions were carried out and recorded using Roche LC480 96 well Real-time PCR machine.

2.2.2.9 Fura-2 videomicroscopy and Ca^{2+} imaging

MEF cells were cultured on 35mm glass-bottom dishes (MatTek #P35GC-0-10-C) and were allowed to grow to about 50% confluency. Prior to experimentation cells were washed twice with 1ml of modified Krebs-Henseleit Buffer (KHB) (120 mM NaCl, 4.8 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5 mM HEPES, 1 mM probenecid). Cells were then loaded with 2 µM fura-2-acetoxymethyl ester (Bio-sciences #F-1201) and incubated for 30 min at 37°C. Cells were then washed twice with 1ml of Ca^{2+}-free KHB (buffered with 0.5mM of EGTA to Ca^{2+}) and

immersed in 1 ml of Ca^{2+}-free KHB. The dishes were then placed on the stage of an Olympus 1X51 inverted fluorescence microscope within an encapsulating incubator (Solent Scientific, Segensworth, UK) maintained at 37 °C. To induce ER calcium discharge, 5 µl of 1mM Ionomycin (Sigma #I3909) was loaded to the culture and alterations in the cytosolic Ca^{2+} levels were detected by exciting the fura-2 loaded cells intermittently by 340 and 380 nm UV light (Cairn Monochromator and 75-W Xenon lamp, Cairn, Faversham, Kent, UK). Emitted light was collected via an Olympus UplanF1 1.3 NA 100x oil-immersion objective, filtered through a dichroic mirror (400 nm cut-off) and recorded using a Hamamatsu ORCA-ER CCD videocamera (Hamamatsu Photonics Ltd., Hertfordshire, UK), set at exposure time of 500 ms per channel. Hardware was controlled and images were acquired using Andor IQ v1.9 software (Andor, Belfast, Northern Ireland). Ratio images were generated and the perimeter of each cell was defined as a region of interest and the mean fura-2 ratio from within this region against time was exported to Microsoft Excel 2003 for further analyses.

2.2.2.10 Flow cytometry for cell surface IL-1R1

HEK293T cells or MEF cells were transfected with IL-1R1 constructs for 48 hours. Then cells were washed one time with ice-cold 1xPBS and detached from plates by PBSE buffer (1xPBS, 50mM EDTA). $1x10^6$ cell aliquots were transferred to each assay tube and span at 1000rpm for 3 minutes. Then the supernatant was taken off and cells were suspended and incubated with mouse anti-IL-1R1 primary antibody diluted in PBSE buffer supplemented with 1%BSA for 45 minutes at room

temperature. After the incubation, cells were washed three times in PBSE buffer. Then the cells were incubated with secondary green-fluorescent anti-mouse antibody (Alexa Fluor 488) for 30 minutes at room temperature. Cells were then washed three more times and then analyzed on a FACScan flow cytometer (Becton Dickinson, Oxford, UK). Results were collected and analyzed by CellQuest and WinMDI softwares.

2.2.2.11 Luciferase assays

HEK293T cell cultures were transfected with wild-type IL-1R1 or IL-1R1 mutants and co-transfected with 100ng NF-κB reporter plasmid (plasmid containing three NF-κB binding elements and a luciferase reporter gene) (Promega) using the calcium phosphate transfection method. If IL-1β treatments are required, cells were serum-starved overnight 24 hours after transfection and then treated with 10nM IL-1β for 6 hours. Generally 48 hours after transfection, cells were harvested in 200 µl passive lysis buffer. Lysates were spun at 13,000 rpm for 10 minutes at 4°C and supernatants were transferred into a fresh eppendorf. Next 20 µl of each lysate was mixed with 100 µl of luciferase substrate (Promega) and the light emission measured as relative light units (RFU) on a luminometer (Turner Designs). Protein concentrations were determined using the BCA method and the RFU/µg of each sample determined. Western Blot analysis for IL-1R1 confirmed expression of transfected IL-1R1 constructs.

2.2.2.12 *In vitro* ubiquitination assay

The peptide arrays which are synthesized by automatic SPOT synthesis on continuous Whatman cellulose membrane using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Rosbot ASS 222 (Intavis Bioanalytical Instruments) are produced and provided by Dr Patrick Kiely. Peptide arrays were spotted with peptides corresponding to the human IL-1R1 C-terminus sequence (301-569) (see **Figure 4.1**). *In vitro* ubiquitination assay was performed using Ubiquitination Kit (#UW9920) from Enzo Life Sciences. Standard reaction setup is listed below. The total reaction volume for immunoprecipitated protein is 50µl and for peptide array is 1ml.

Component	Target-Ub	Target Ubiquitin –ve control	TE +ve control	TE –ve control
		volume / µL		
dH_2O	14	11.5	21.5	19
10x Ubiquitinylation Buffer	5	5	5	5
IPP (100U/mL)	10	10	10	10
DTT (50mM)	1	1	1	1
Mg-ATP (0.1M)	2.5	-	2.5	-
EDTA (50mM)	-	5	-	5
20x E1 (2µM)	2.5	2.5	2.5	2.5
10x E2 (0.5mg/mL, 18-28µM)	5	5	5	5
20x E3 (2µM)	2.5	2.5	-	-
10x Target protein (10µM)	5	5	-	-
20x Bt-Ub (50µM)	2.5	2.5	2.5	2.5

For immunoprecipitated protein, assay components were added to 0.5ml tubes. After gentle mix, the tubes were incubated at 37°C for 60 min under gentle shaking. After reaction, the substrate-conjugated beads were washed twice with covalent buffer. Then the beads were loaded with SDS sample loading buffer and were boiled for 5 min. After spinning for 2 min at 1000rpm, the supernatants were loaded on gel and analysed by Western Blotting.

For *in vitro* ubiquitination assay of IL-1R1 arrays, the mixed reaction components were sealed with the peptide arrays in plastic bags and then were incubated at 37°C

for 60 min under gentle shaking. After reaction, IL-1R1 peptide arrays were washed in 0.2M NaOH stripping buffer to remove non-covalent binding. Ubiquitination status of the IL-1R1 peptide array was revealed by Western Blot analysis and probing the membranes with anti-ubiquitin antibody.

Chapter 3:

RESULTS

Presenilins are novel substrates of TRAF6-mediated ubiquitination

Introduction:

An increasing number of type-I integral membrane proteins has been reported as proteolytic substrates of γ-secretase cleavage, including Notch receptor, APP and IL-1R1 which is reported as a γ-secretase substrate previously by our group (Elzinga et al., 2009b). Thus studying the regulation of γ-secretase activity is considered very important in understanding the pathogenesis of AD, the role of Notch signalling in cell differentiation and other γ-secretase substrates involved events. Presenilins, as the catalytic core of γ-secretase complex, are tightly regulated to maintain the homeostasis of the protein levels and functional γ-secretase complexes (Thinakaran et al., 1997). Presenilin holoproteins undergo endoproteolysis and the NTF and CTF associate as stable heterodimers (Thinakaran et al., 1996). In addition to endoproteolysis, presenilins are also regulated by diverse posttranslational modifications including phosphorylation (Kirschenbaum et al., 2001a, b; Twomey and McCarthy, 2006) and ubiquitination (Li et al., 2002; Massey et al., 2005). These modifications are not only essential for the stability and activation of presenilins, but are also important for the protease assembly and activity of γ-secretase complexes. For instance, SEL-10 was shown to interact with and enhance PS1 ubiquitination, and alter the cellular levels of PS1 holoprotein and its NTF/CTF heterodimers (Li et al., 2002). Our group have also reported the association between PS1 and TRAF6 previously (Powell et al., 2009). The interaction between PS1 and TRAF6 was shown to be ligand-dependent and disruption of this interaction antagonizes the γ-secretase cleavage of a TRAF6-regulated receptor, $p75^{NTR}$.

TRAF family proteins are critical regulators for the signal transduction of a wide variety of receptors (Chung et al., 2002a). For example, TRAF6 is an essential adaptor protein for IL-1R1/TLR4 signalling transduction to the activation of NF-κB and MAPK. TRAF6 possesses E3 ligase activity, as well as TRAF2 and TRAF5 among the TRAF family members. TRAF6 has been shown to facilitate a diversity of signalling pathways by catalysing different types of ubiquitination. TRAF6 facilitates K63-linked polyubiquitination at five different lysine residues within NEMO which leads to activation of IKK and NF-κB (Sebban-Benin et al., 2007b). Unlike Lys48-linked polyubiquitination, which targets proteins for proteasomal degradation, Lys63-linked polyubiquitination does not degrade targeted proteins, but activates signalling pathways. TRAF6 also promotes lysine-6, lysine-27 and lysine-29 linked ubiquitination of amino terminal fragments of huntingtin protein and involves in Huntington's disease pathogenesis (Zucchelli et al., 2011).

The autoubiquitination of TRAF6 is reported to be essential for TRAF6 activation in certain signalling pathway. TAB2 is reported to facilitate ubiquitination of TRAF6 through its CUE domain which is also indispensable for TRAF6 interaction with IKK (Kishida et al., 2005). The CUE domain was firstly discovered as an ubiquitin-binding domain targeting ubiquitinated protein to degradation pathways (Ponting, 2000b). Later on, CUE domain was also revealed to promote the ubiquitination of the proteins that contain it (Shih et al., 2003a).

TRAF6 is a critical regulator for RANK signalling. TRAF6-mediated RANK signalling is essential for the differentiation and activation of osteoclast (Armstrong et al., 2002).

Deficiency of TRAF6 results in severe osteopetrosis and disrupted osteoclast functions such as bone resorption and cytoskeletal organization (Lomaga et al., 1999a; Armstrong et al., 2002). Interestingly, inhibition of ER Ca^{2+} signalling by blocking of the ER calcium ATPase, which interacts with PS1, results in reduction of osteoclastic survival and bone resorption (Mentaverri et al., 2003). Moreover, deficiency of PS1 in mice was reported to cause skeletal defects (Shen et al., 1997).

In this study, we attempted to further characterise and determine the relevance of the interaction between presenilins and TRAF6. Firstly, we started with confirming the interaction between presenilins and TRAF6 and investigating the possible regulations mediated by TRAF6. Secondly, as TRAF6 is an E3 ligase, we sought to test if presenilins are novel substrates of TRAF6-mediated ubiquitination. Thirdly, as SEL-10-mediated PS1 ubiquitination has been shown to regulate PS1 protein levels and APP processing (Li et al., 2002), we were planning to investigate the functional significance of TRAF6 mediated regulation of presenilins in terms of presenilin stability, γ-secretase activity and γ-secretase independent functions of presenilins. Finally, we attempted to study the CUE domain of presenilins as a novel presenilin ubiquitin-binding domain and reveal the function of this CUE domain.

3.1 TRAF6 increases transcriptional and cellular levels of presenilins

3.1.1 PS1 and PS2 interact with TRAF6

Our group has previously shown that PS1 contains a putative TRAF6-binding domain by sequence analysis of known TRAF6 interacting proteins. It was also demonstrated that PS1 interacts with TRAF6, in part via a conserved consensus motif within the hydrophilic loop of PS1, and that mutagenesis of two sites (Pro374 and Glu376) within this motif antagonizes the interaction between PS1 and TRAF6 (Powell et al., 2009). In this study, we began by confirming the interaction of PS1 or PS2 with TRAF6. HEK293T cells were transfected with expression constructs encoding untagged PS1 or PS2 and co-transfected with FLAG-tagged TRAF6 or TRAF6 dominant negative mutant (TRAF6DN) lacking the catalytic N-terminus RING domain and zinc finger domains. Consistent with previous studies, immunoprecipitation of FLAG-TRAF6 quantitatively co-precipitated PS1 **(Figure 3.1A)** and PS2 **(Figure 3.1B)** and reciprocal immunoprecipitation also revealed the same interactions. Interaction between TRAF6DN and presenilins suggests that the binding motif of TRAF6 for presenilins localises in the TRAF6 C-terminus domain. Western blot analysis of all cell lysates revealed equal protein expression levels. Interestingly, co-expression of TRAF6 with PS1 or PS2 enhanced the immunodetection of full length PS1 and PS2 **(Figure 3.1 A lane 3 and B lane 2)**.

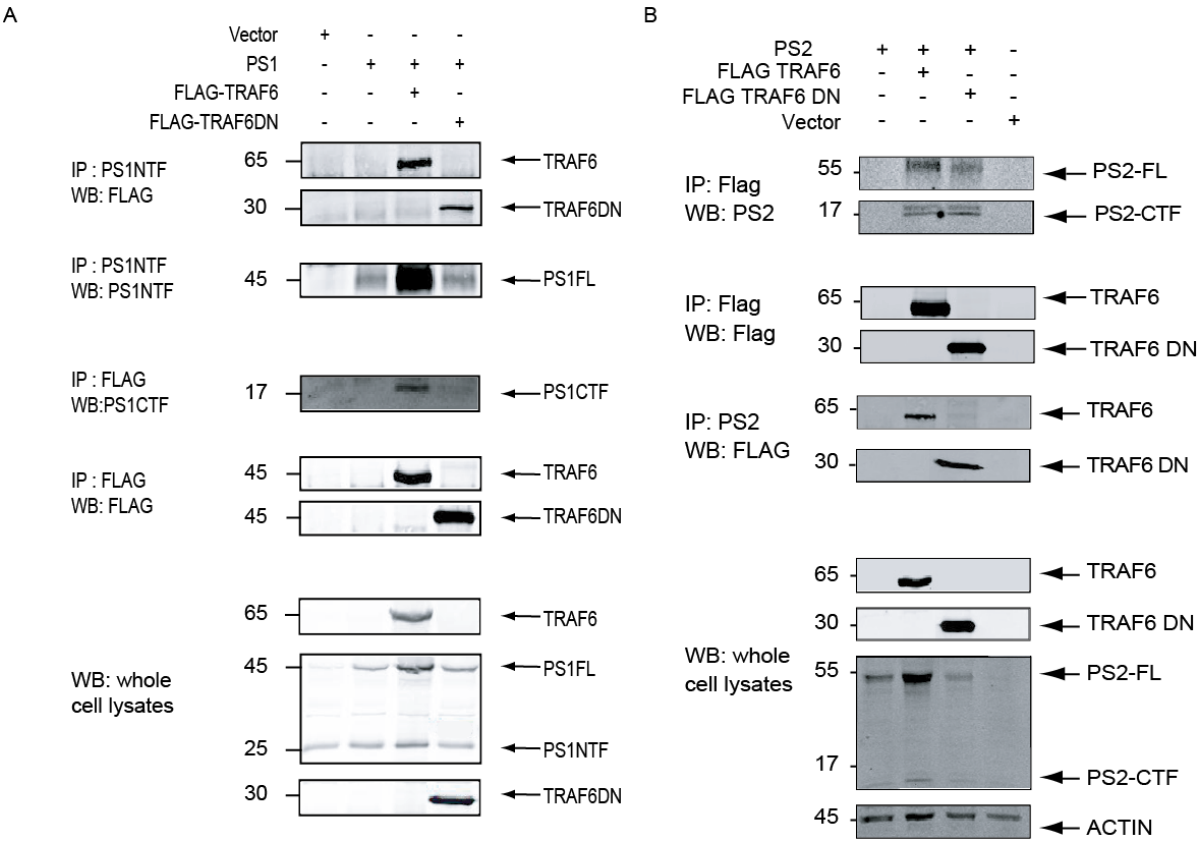


Fig 3.1 PS1 and PS2 interact with TRAF6. HEK293T cells were transiently transfected with wild type Presenilins and co-transfected with FLAG tagged TRAF6 or TRAF6 DN. Thirty-six hours post-transfection extracts were prepared and immunoprecipitated (IP) for presenilins or FLAG-TRAF6 and then analysed for FLAG-TRAF6 or presenilins respectively by Western Blot. Co-precipitations of PS1 (**A**) or PS2 (**B**) with TRAF6 or TRAF6 DN were detected by immunoblotting with anti-presenilin or anti-FLAG antibodies, as indicated. Western blotting of whole cell lysates confirmed the expression of all transfected constructs. Data presented are representative of a typical experiment (n=3).

3.1.2 TRAF6 increases presenilin levels in a dose-dependent manner

Having shown increased immunodetection of PS1 and PS2 full length with co-transfection of TRAF6, we next investigated whether increased presenilin immunodetection was dose-dependent with increased expression of TRAF6. HEK293T cells were transfected with PS1 or PS2 and co-transfected with increasing amounts of TRAF6. Cell lysates were immunoblotted with anti-PS1 NTF **(Figure 3.2A)** or anti-PS2 CTF **(Figure 3.2B)** antibodies respectively. Consistent with data presented in Figure 3.1, co-expression of PS1 with increasing concentration of TRAF6 resulted in a dose-dependent increase in detectable levels of PS1 (**Figure 3.2A**) and PS2 (**Figure 3.2B**) full length. Densitometry was used to measure the relative levels of PS1 and PS2 detected by Western blot. All levels of presenilins measured were normalised to the corresponding β-actin levels and expression levels of presenilin full length and fragments without TRAF6 were set as control (1 fold). Importantly, when co-transfected with 1.6μg TRAF6, immunoreactivity of full length PS1 was increased by ~3 fold and immunoreactivity of PS2 full length was increased by ~4 fold. PS1 and PS2 fragments were also increased by TRAF6 but only to the comparably lesser levels, suggesting a preferential increase in PS1 full length levels. Dosing amounts of TRAF6 were detected by immunoblotting with anti-FLAG antibody.

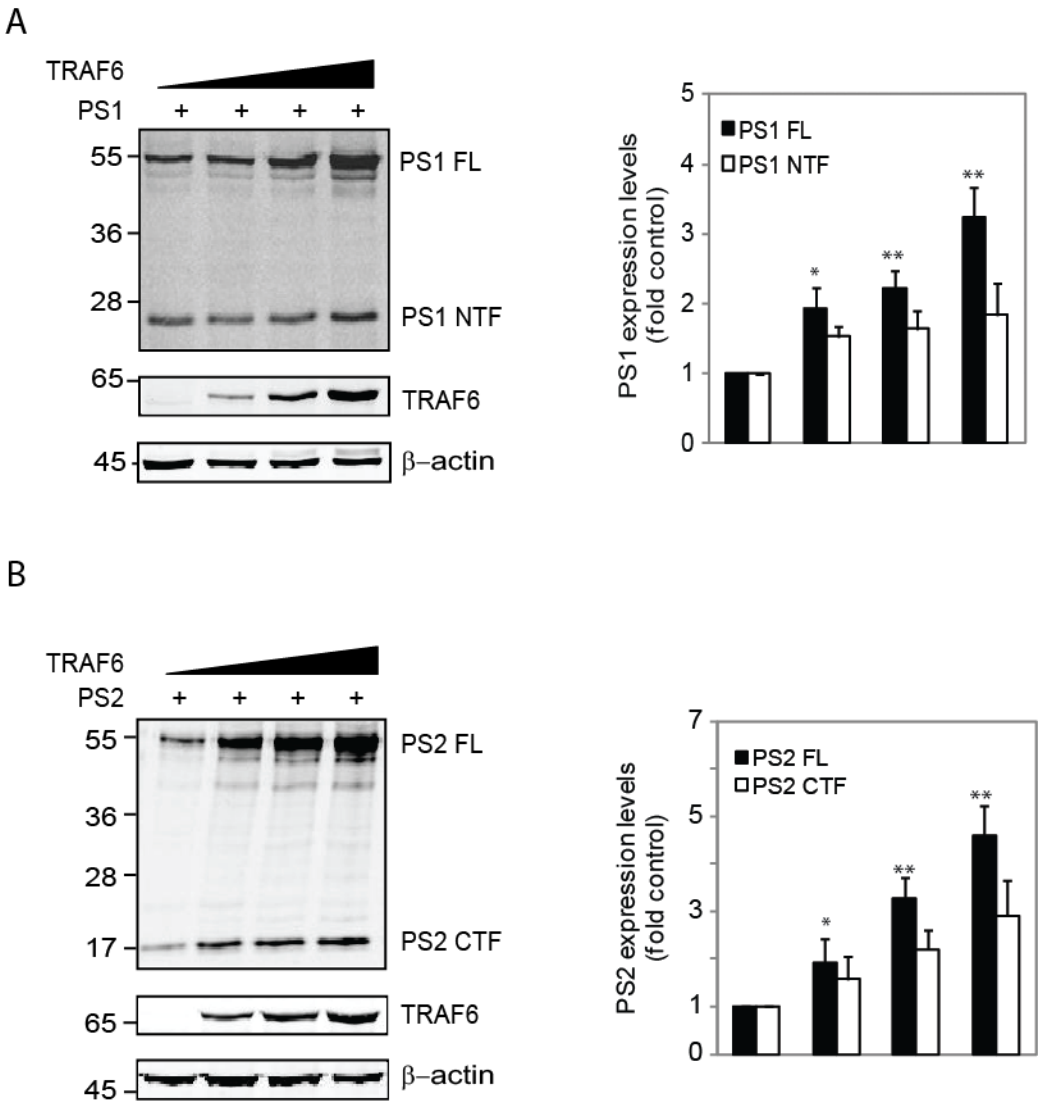


Figure 3.2 TRAF6 enhances immunodetection of PS1 and PS2. HEK293T cells were transfected with expression constructs for PS1 (0.8µg) or PS2 (0.8µg) and co-transfected with increasing amounts of TRAF6 (0-1.6µg). Equal amount of plasmid was transfected in each culture by complement with empty vector. Cell lysates were analyzed by Western blot with anti-PS1 NTF, anti-PS2 CTF, anti-FLAG and anti-β-actin antibodies. PS1 and PS2 full length and fragment immunoreactivity was measured by densitometry and is shown as mean + SEM. n=4.

3.1.3 Effect of TRAF6 on presenilin immunodetection requires TRAF6 E3 ligase activity

TRAF6 possesses E3 ligase activity and it requires its E3 ligase activity to maintain efficient signal transduction (Lamothe et al., 2007a). To investigate whether TRAF6 E3 ligase activity is required for the effect on PS1 and PS2 immunodetection, we used a dominant negative TRAF6 mutant (TRAF6DN), which lacks the RING and zinc finger domains (TRAF6 300-524) and is defective in TRAF6 E3 ligase activity (Lamothe et al., 2008). HEK293T cells were transfected with wild type PS1 and co-transfected with increasing amounts of TRAF6 or TRAF6-DN. Cell lysates were analysis by Western blotting with anti-PS1 CTF antibody. Consistent with our previous data, co-expression of TRAF6 with PS1 caused an increase in immunodetection of PS1. In contrast, co-expression of TRAF6-DN with PS1 did not increase PS1 immunodetection, but decreased immunodetection of PS1 in a dose-dependent manner **(Figure 3.3A)**. Dosing expression of TRAF6 and TRAF6-DN were confirmed by Immunoblotting with anti-FLAG antibody.

Having shown that the effect of TRAF6 on PS1 immunodetection may require its E3 ligase activity, we next utilised a series of TRAF6 mutant constructs to confirm our hypothesis; the TRAF6 single RING finger cysteine mutant TRAF6C70A is defective in TRAF6 substrate ubiquitination, while the ubiquitin acceptor site mutant, TRAF6K124R is defective in TRAF6 autoubiquitination (Lamothe et al., 2007a). HEK293T cells were transfected with PS1 or PS2 and co-transfected with TRAF6, TRAF6-DN, TRAF6K124R and TRAF6C70A. Co-expression of either TRAF6-DN or TRAF6C70A, mutants that are defective in TRAF6 substrate ubiquitination activity,

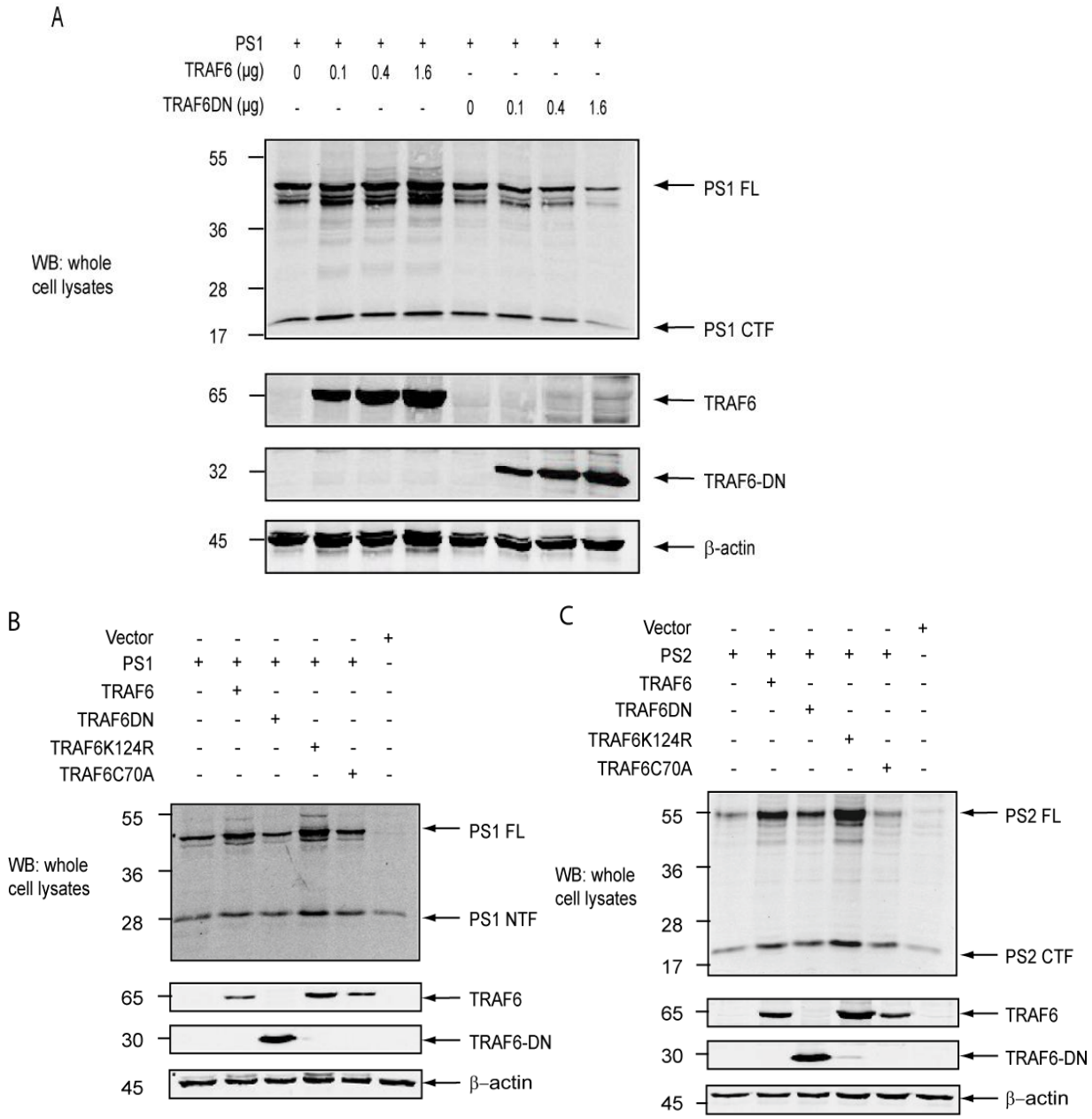


Figure 3.3 Effect of TRAF6 on presenilin immunodetection requires TRAF6 E3 ligase activity. **(A)** HEK293 cells were transfected with expression constructs for PS1 and co-transfected with increasing amounts of TRAF6 or TRAF6-DN. Cell lysates were analyzed by Western Blot with anti-PS1 CTF, anti-FLAG and anti-β-actin antibodies. **(B)** HEK293 cells were transfected with PS1 and co-transfected with TRAF6, TRAF6-DN, TRAF6K124R and TRAF6C70A. Cell lysates were analyzed by Western Blot with anti-PS1 NTF, anti-FLAG and anti-β-actin antibodies. **(C)** HEK293 cells were transfected with PS2 and co-transfected with TRAF6, TRAF6-DN, TRAF6K124R and TRAF6C70A. Cell lysates were analyzed by Western Blot with anti-PS2 CTF, anti-FLAG and anti-β-actin antibodies. Experiment was repeated at least three times with the similar results.

failed to enhance levels of PS1 and PS2 **(Figure 3.3B and C lane 3 and 5)**. In comparison TRAF6K124A, defective in TRAF6 autoubiquitination, retained its ability to increase PS1 and PS2 detectable levels **(Figure 3.3B and C lane 4)**. Thus it suggests that levels of PS1 and PS2 are enhanced following over-expression of TRAF6 with catalytically active E3 ligase activity.

3.1.4 TRAF2, TRAF5 and TRAF6 preferentially enhance presenilin levels

As TRAF6 is not the only TRAF protein that contains a RING domain and functions as an E3 ligase (Lee and Lee, 2002), we selected other TRAFs to explore if enhanced immunoreactivity of presenilin is unique to TRAF6 or the effect is common among TRAF family members. HEK293T cells were transfected with PS1 or PS2 and co-transfected with FLAG-tagged TRAF2, TRAF2-DN, TRAF3, TRAF4, TRAF5 and TRAF6. Though all TRAF family protein appeared to induce enhanced immunodetection of PS1 and PS2, co-expression with TRAF2, TRAF5 and TRAF6 consistently increased full length and fragment detections of PS1 and PS2 **(Figure 3.4A and B)**. TRAF3 and TRAF4 only moderately enhanced presenilin detections. But to our surprise, TRAF2-DN also induced increased presenilin levels which may be explained as TRAF2-DN could still be recruited with TRAF6 as an adaptor even without its catalytic RING domain. But this assumption needs to be further confirmed. Redundancies between TRAF family members have been reported before. For example, depletion of both TRAF2 and TRAF5 are required for abolishment of TNF-induced NF-κB activation (Tada et al., 2001). Similarly, deficiency of both TRAF2 and TRAF6 is necessary for abrogating CD40-mediated NF-κB activation and TRAF2 and TRAF6 associate at the CD40 complex (Davies et al., 2005; Ellison et al., 2006). For TLR signalling, mutations

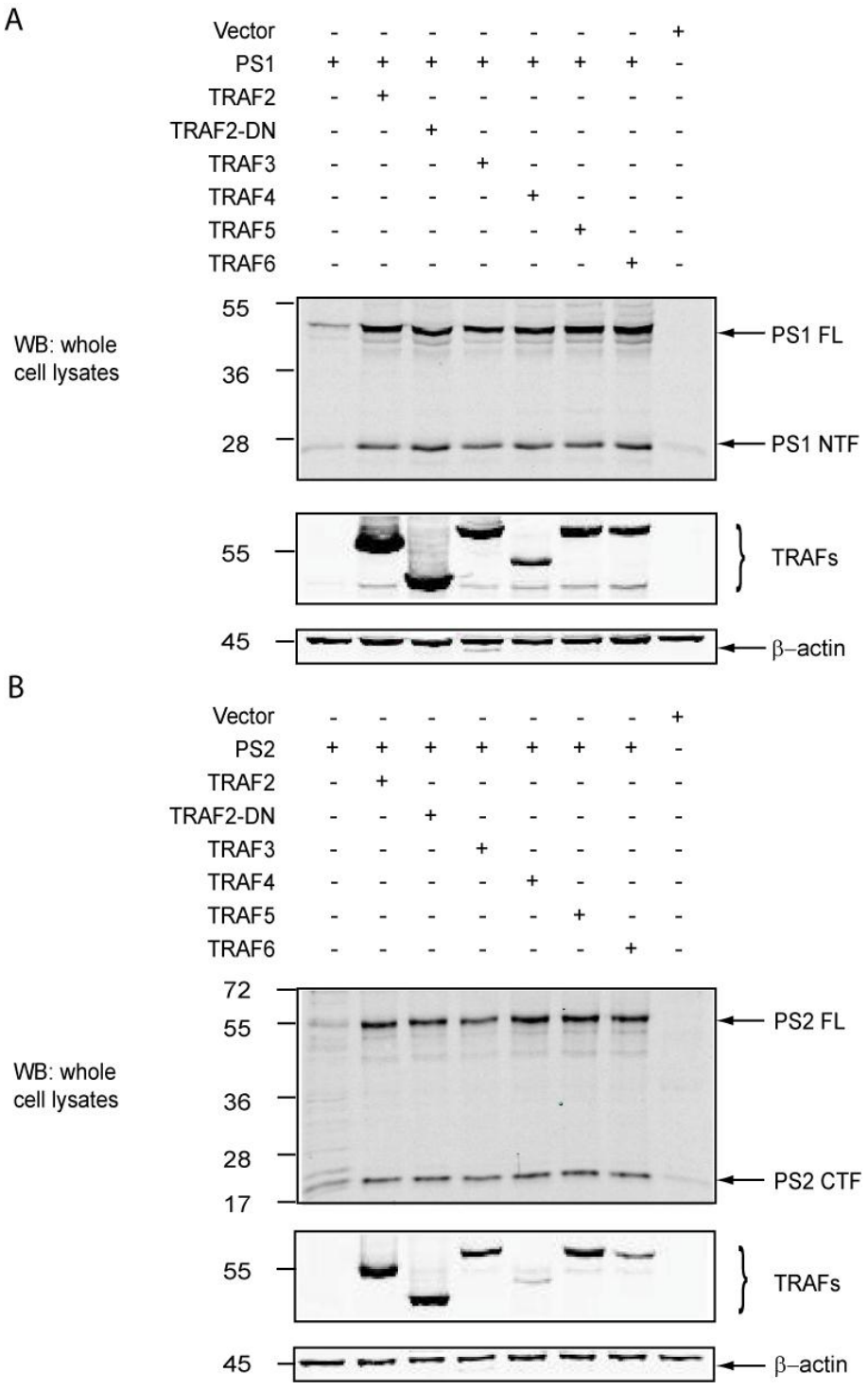


Figure 3.4 TRAF2, TRAF5 and TRAF6 preferentially enhance immunoreactivity of the presenilins. HEK293 cells were transfected with PS1 **(A)** or PS2 **(B)** and co-transfected with TRAF2, TRAF2DN, TRAF3, TRAF4, TRAF5 and TRAF6. Thirty-six hours post-transfection cell lysates were analyzed by Western Blotting with anti-PS1NTF or anti-PS2CTF antibodies. Blots were re-probed with anti-FLAG and anti-β-actin antibodies to show equivalent protein expression levels.

of both TRAF2 and TRAF6 binding sites in TRIF are required to abrogate type I IFN induction (Sasai et al., 2010). Collectively, redundancies between TRAF family proteins, especially between TRAF2, TRAF5 and TRAF6 have been observed by different groups and we investigated further at the level of presenilin regulations.

3.1.5 TRAF6 increases PS1 mRNA level through JNK activation.

Previous studies showed that expression of PS1 is regulated by c-jun-NH2-terminal kinase (JNK) activity (Lee and Das, 2008). Also auto-ubiquitination of TRAF6 could induce JNK activation and inhibition of TRAF6 polyubiquitination leads to suppressed JNK activation (Loniewski et al., 2007; Chen et al., 2012). To confirm this hypothesis and to determine if increased PS1 levels is caused partially from TRAF6 induced JNK activation, we applied an inhibitor for JNK activation, SP600125 and investigate its effects on both PS1 expression and transcription. HEK293T cells were transfected with PS1 and co-transfected with TRAF6 or TRAF6 C70A mutant. Twenty-four hours after transfection, cells were treated with SP600125 (40 μM) in serum-free medium for 12 hours. Cell extracts were then prepared and analysed for PS1 protein levels and JNK activity by Western blotting. As shown in **Figure 3.5A**, SP600125 decreased basal phosphorylation of JNK and overexpression of TRAF6 induced JNK activation in an E3 ligase activity dependent manner which could be attenuated by the JNK inhibitor. Inhibition of JNK activity also decreased full-length protein level of PS1 and attenuated TRAF6-induced increases in the expression of PS1. Furthermore, cells expressing PS1 or co-expressing PS1 and TRAF6 were treated with SP600125 and total RNA was extracted from these cells and subjected to reverse transcription PCR and Real-time PCR. We showed that PS1 mRNA level was decreased by inhibition of JNK **(Figure 3.5B**). TRAF6 increased the mRNA level of PS1 which is reversed

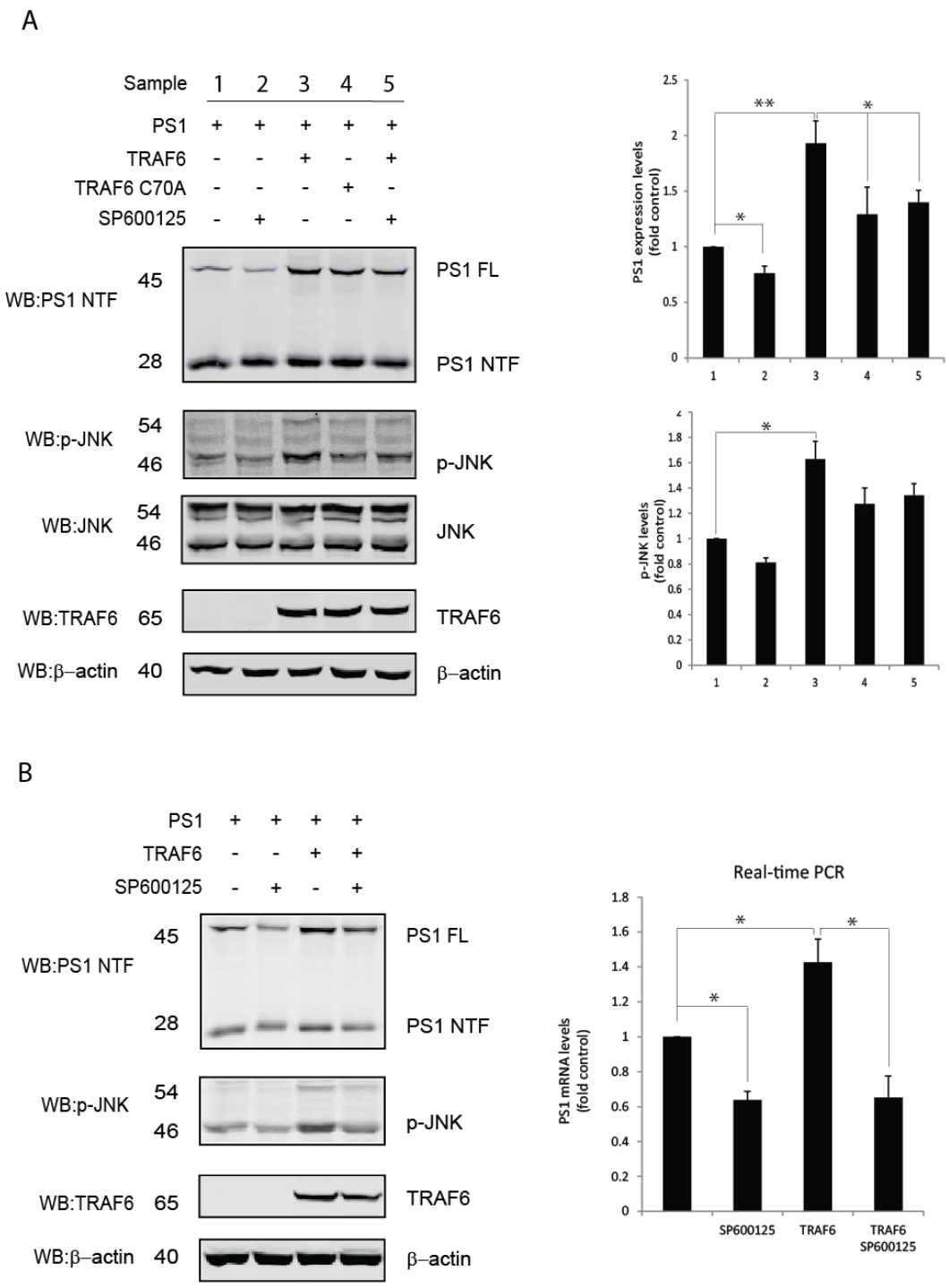


Figure 3.5 TRAF6 increases PS1 mRNA level through JNK activation. (A) HEK293T cells were transfected with PS1 alone or co-transfected with TRAF6 or TRAF6 C70A mutant. 24 hours after transfection, cells were treated with 40 µM SP600125 in serum-free medium for 12 hours. Cell extracts were then prepared and analysed for PS1 protein levels and JNK activity by Western blotting using PS1 NTF and phosphorylated JNK antibodies. Levels of PS1 full-length and p-JNK were measured by densitometry and shown as mean + SEM. **(B)** HEK293T cells were transfected with PS1 alone or co-transfected with TRAF6 and treated with 40 µM SP600125 in serum-free medium for 12 hours. Then total RNA was extracted from these cells and subjected to reverse transcription PCR and Real-time PCR. Levels of PS1 mRNA were shown as relevant 2^(-ddCt) values normalised to the levels of the house keeping gene GAPDH.

by SP600125, the JNK activation inhibitor, suggesting that TRAF6 regulates mRNA level of PS1 by inducing activation of JNK.

3.1.6 Knock-out of TRAF6 decreases endogenous presenilin levels

We also investigated effect of TRAF6 deficiency on presenilin levels by comparing the level of PS1 and PS2 in the MEF WT cells and the MEF TRAF6 knock-out (TRAF6-/-) cells. Control MEF cells and $TRAF6^{-/-}$ MEF cells were lysed and subjected to BCA assay to determine the protein concentration of the lysates. To achieve clear comparison, increasing amounts of cell lysates from each cell line were loaded and blots were subjected to PS1 NTF and PS2 CTF antibodies. Endogenous full-length presenilins are too faint to be detected, but we showed that levels of endogenous presenilin fragments are reduced in TRAF6 knockout MEF cells by about 30-40% **(Figure 3.6A)**. Additionally, we attempted to restore TRAF6 level by exogenous expression of TRAF6 in $TRAF6^{-/-}$ MEF cells **(Figure 3.6B)**. Overexpression of TRAF6 partially rescued the levels of PS1NTF and PS2CTF. Expression of exogenous TRAF6 in $TRAF6^{-/-}$ cells and endogenous TRAF6 in control MEF cells were confirmed by blotting with anti-TRAF6 antibody. Collectively, these data suggest that TRAF6 plays important role in the abundance of presenilins. However, knock-out of TRAF6 did not eliminate presenilins and previously we showed that other TRAF protein also increase presenilin levels, suggesting the potential functional redundancy among TRAF family proteins in regulating presenilin levels.

3.1.7 TRAF6 alters turnover of PS1

Having shown that TRAF6 enhances the immunodetection of presenilins and increase PS1 transcription through JNK activation, we next examined whether or not

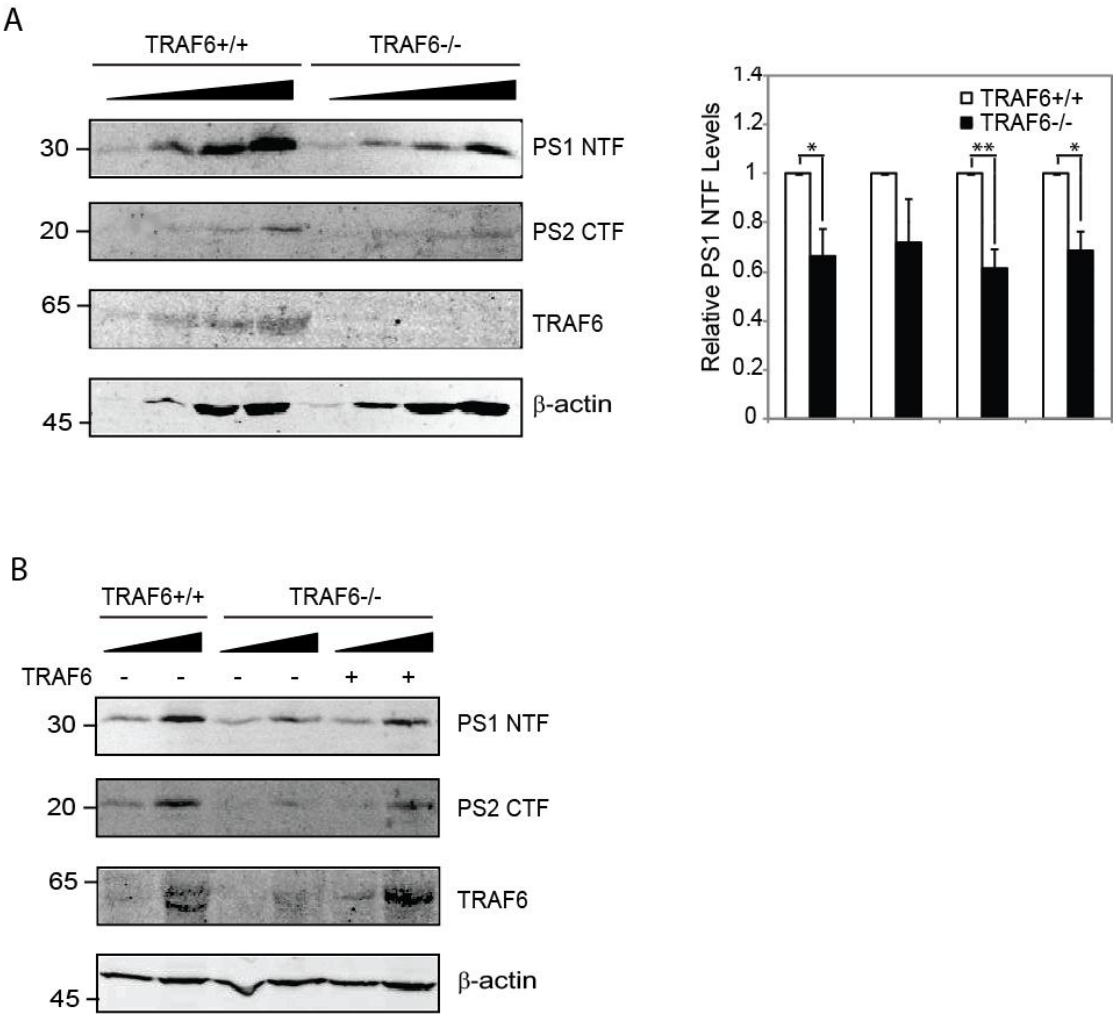


Figure 3.6 Knock-out of TRAF6 decreases endogenous presenilin levels. (A) Increasing amount (5-40µg) of cell lysates from both wild type MEF cells and TRAF6 knock-out MEF cells were subjected to Western blot analysis. Levels of PS1 NTF and PS2 CTF were revealed by blotting with PS1 NTF and PS2 CTF antibodies. Knock-out of TRAF6 was confirmed by probing with anti-TRAF6 antibody. **(B)** TRAF6-/- MEF cells were transfected with TRAF6 for 36 hours and then were harvested and subjected to Western blot together with untransfected cell lysates from wild-type MEF cells and TRAF6-/- MEF cells. Levels of PS1NTF and PS2CTF were revealed by blotting with PS1 NTF and PS2 CTF antibodies. Expression of transfected TRAF6 in TRAF6-/- cells and endogenous TRAF6 in WT MEF cells were confirmed by blotting with anti-TRAF6 antibody.

the effects of TRAF6 on PS1 protein levels involved posttranslational modification of PS1. To test this we examined PS1 protein half-life using the protein synthesis inhibitor, cycloheximide. HEK293T cell cultures were either transfected with PS1 alone, or co-transfected with TRAF6. Twenty-four hours post-transfection, cultures were supplemented with 20μg/ml cycloheximide. Cell cultures were then harvested 0, 1, 2, 4, 8, 12 and 24 hours after cycloheximide treatment according to previous reports that the half-life of full length PS1 is ~50 minutes and the half-life of the NTF/CTF fragments is ~24 hours (Ratovitski et al., 1997; Zhang et al., 1998). Cell lysates were immunoblotted with anti-PS1 NTF antibody to reveal the levels of PS1 full-length and NTF **(Figure 3.7)**. The cellular level of full length PS1, when co-expressed with TRAF6, declined relatively slowly especially after 12 hours of cycloheximide treatment. Densitometry was used to measure the immunodetection of PS1 full length. All PS1 full length levels were normalized to the corresponding tubulin levels and protein levels when cycloheximide was added were set as control (1 fold). Densitometry analysis showed that the half-life of PS1 full length is ~30 minutes and TRAF6 increased the half-life of PS1 full length by ~1 hour **(Figure 3.7 lower panel)**. After 12 hours cycloheximide treatment, still ~20% of PS1 full length was detected with co-expression of TRAF6. Fragments of PS1 were more stable than full length and no significant difference in PS1 NTF was observed between samples with and without TRAF6. TRAF6 appeared to alter PS1 turnover, suggesting that TRAF6 also induces posttranslational modifications of PS1.

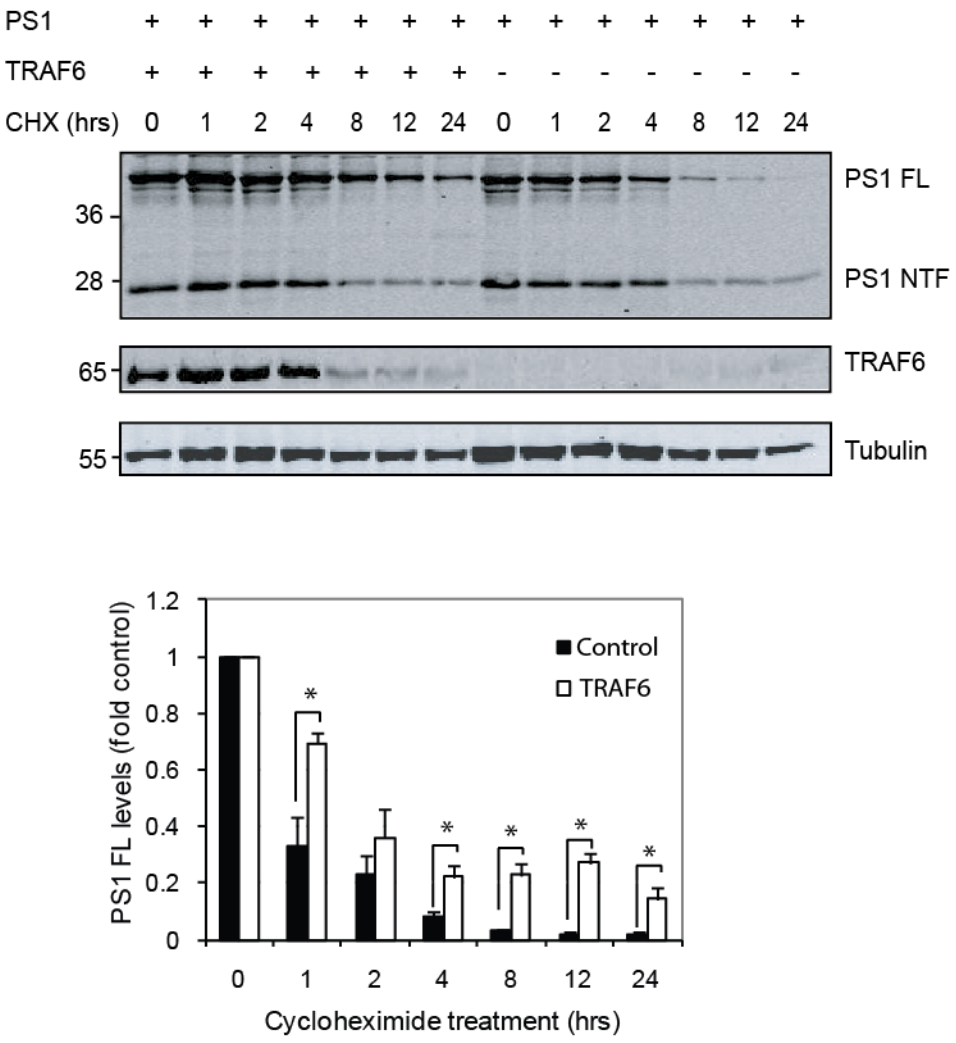


Figure 3.7 TRAF6 alters turnover of PS1. HEK293T cells were transiently transfected either with PS1 alone, or co-transfected with FLAG-TRAF6. Twenty-four hours after transfection, cell cultures were incubated with cycloheximide (20µg/ml) for 0, 1, 2, 4, 8, 12 and 24 hours respectively. Cell lysates were analyzed for PS1 immunodetection by immunoblotting with anti-PS1 NTF and anti-FLAG antibodies. β-tubulin levels were also measured as loading control. Densitometry was used to measure levels of PS1 full length (FL) with blots probed with anti-PS1 NTF antibodies. Bars presented are the mean + SEM of four independent experiments. The differences between two groups are statistically significant ($p<0.05$).

3.2 Identification and characterisation of presenilin CUE domain

3.2.1 Identification of CUE domain and its role in presenilin endoproteolysis

The CUE domain was initially identified as a ubiquitin-binding motif in a yeast hybrid screen and named for the yeast Cue1p protein, which recruits the ubiquitin-conjugating enzyme Ubc7p to the ER, where it is essential for the degradation of misfolded proteins (Biederer et al., 1997; Shih et al., 2003b). Since then, the CUE domain is reported to contribute to the stability and specificity of the CUE-ubiquitin complex, and moreover that CUE domain containing proteins play an important role in stabilizing its binding partner (Kang et al., 2003; Zhang et al., 2007). By sequence analysis of reported CUE domain-containing proteins with the amino acid sequences of PS1 and PS2, our group has identified a previously uncharacterised putative CUE domain in PS1 (271-310) and PS2 (277-316) **(Figure 3.8A)**. The critical Phe/Pro and Val/Ser motifs in PS1 and PS2 are also conserved across other species **(Figure 3.8B).** As the putative CUE domain is localized in the cytosolic loop of PS1 and PS2 and it also contains the human PS1 and PS2 endoproteolytic cleavage sites (Met292/Val293 and Met298/Val299) (Thinakaran et al., 1996; Jacobsen et al., 1999), we next examined the role of the putative CUE domain in presenilin endoproteolysis by mutagenesis of four single points in the CUE domain or deletion of the whole CUE domain (PS1 271-310/ PS2 277-316) from PS1 and PS2. PS1/PS2-deficient mouse embryonic fibroblast (PS1/PS2$^{-/-}$ MEF) cell lines were transfected with expression constructs directing the synthesis of wild type PS1, biologically inactive transmembrane aspartate mutant PS1D257A/D385A, CUE domain deletion mutant PS1ΔCUE, single CUE domain point mutants PS1S310A, PS1V309A, PS1F283A,

A

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Human TAB2   (8-45)     IDFQVLHDLRQKFPEVPEVVVSRCMLQNNNNLDACCAVLS
human TAB3   (8-45)     LDIQVLHDLRQRFPEIPEGVVSQCMLQNNNNLEACCRALS
human Tollip(228-270    CSEEDLKAIQDMFPNMDQEVIRSVLEAQRGNKDAAINSLL
human CUEDC2(133-180)   ELLPGVDVLLEVFPTCSVEQAQWVLAKARGDLEEAVQMLV
yeast VpS9   (408-450)  ERKDTLNTLQNMFPDMDPSLIEDVCIAKKSRIGPCVDALL
human PS1    (271-310)  LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVS
human PS2    (277-316)  LVETAQERNEPIFPALIYSSAMVWTVGMAKLDPSSQGALQ
     Consensus                    FP                        LL
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B

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Human      PS1     LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVS
Chimp      PS1     LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVS
Dog        PS1     LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRKVS
Mouse      PS1     LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRKVS
Rat        PS1     LVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVP
Chicken    PS1     LVETAXERNETLFPALIYSSTMVWLVNMAEEDPEGQRKAS
Zebrafish PS1      LVETAQERNEAIFPALIYSSTMVWLFNMADSAETRNNSSH
C.elegans PS1      LVETAQERNEPIFPALIYSSGVIYPYVLVTAVENTTDPRE

  Consensus        L           FP                        VS
```

Figure 3.8 PS1 and PS2 contain a putative CUE domain. (A) alignment of amino acid sequences of the CUE domain from human TAB2, TAB3, Tollip, CUEDC2, PS1, PS2 and *S.cervisiae* Vps9. The important FP and di-Leu motifs are highlighted in red and yellow, while discrepancies in sequences are depicted in green. **(B)** PS1 CUE domain is conserved across species. Sequences of PS1 hydrophilic loop domain across different species were aligned. PS1 CUE domain consensus is conserved across all species examined. Alignments were carried out with AlignX software.

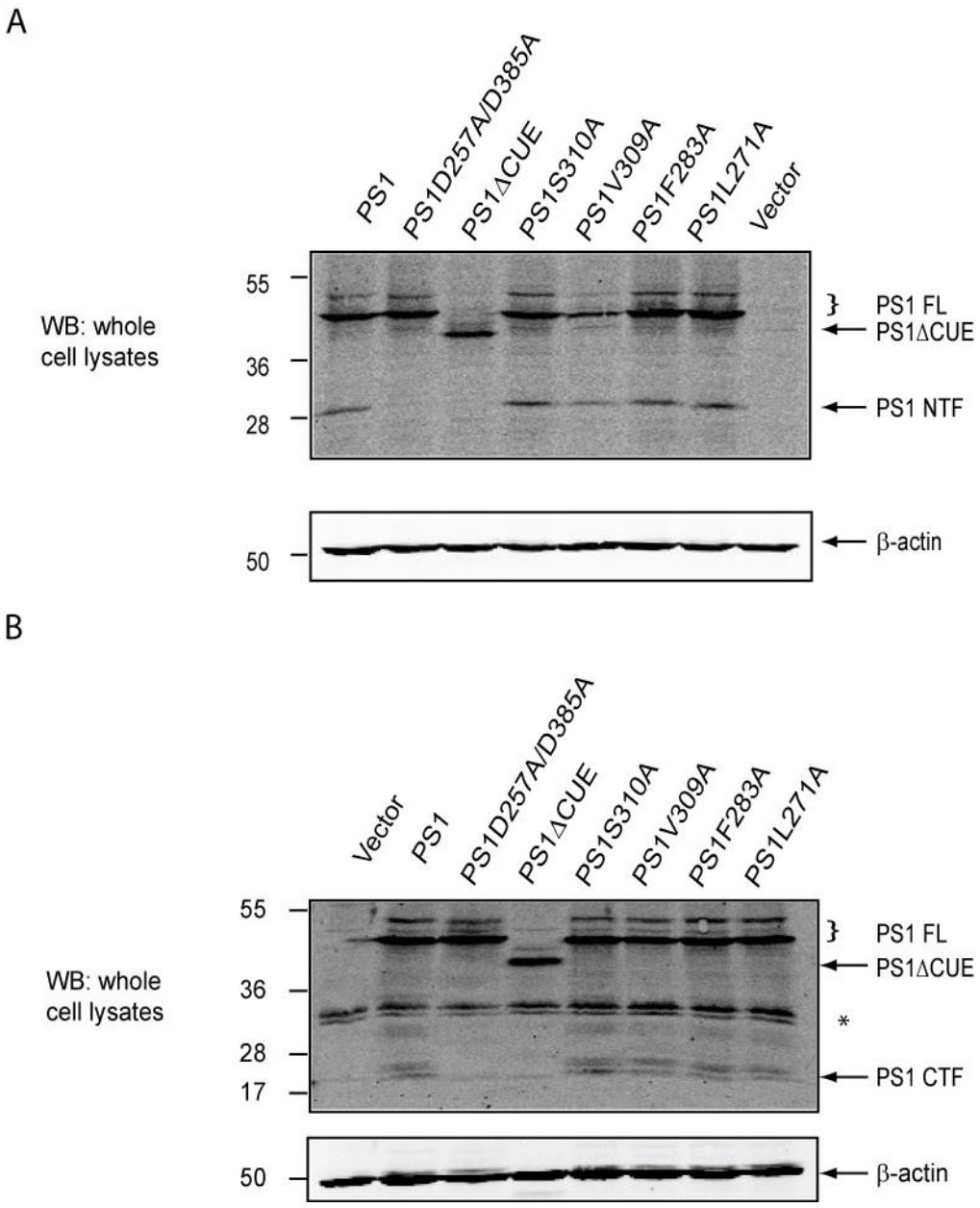

Figure 3.9 Endoproteolysis of PS1 is not altered by single point mutation of the critical CUE domain motifs. PS1/PS2$^{-/-}$ MEF cell cultures were transiently transfected with expression constructs encoding wild type PS1, PS1 D257A/D385A, PS1 CUE domain deletion mutant (PS1ΔCUE), PS1 S310A, PS1 V309A, PS1 F283A or PS1 L271A. Thirty-six hours post-transfection cell lysates were analyzed by SDS-PAGE gel and immunoblotted with anti-PS1 NTF **(A)**, anti-PS1 CTF **(B)** and β-actin antibodies respectively. Asterisk indicates a series of non-specific bands.

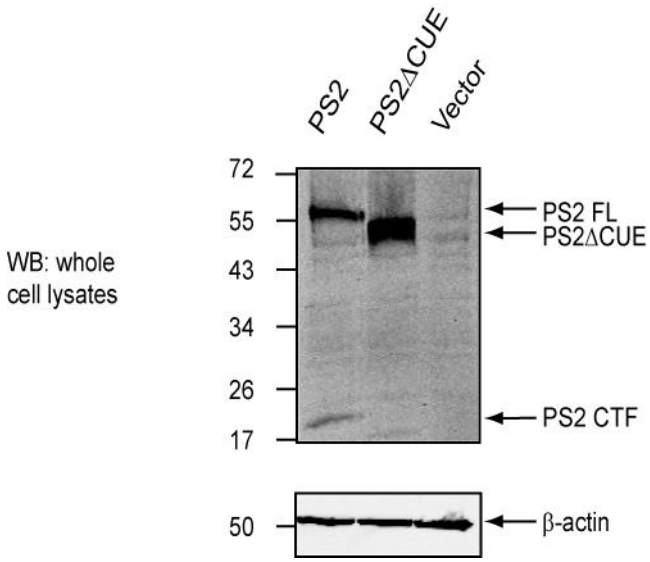

Figure 3.10 Deletion of the CUE domain prevents the endoproteolysis of PS2. PS1/PS2$^{-/-}$ MEF cell cultures were transiently transfected with expression constructs encoding wild type PS2 and PS2 CUE domain deletion mutant (PS1ΔCUE). Thirty-six hours post-transfection cell lysates were analyzed by SDS-PAGE gel and immunoblotted with anti-PS2 CTF and β-actin antibodies. Experiments were repeated at least three times with similar results.

PS1L271A, wild type PS2 or PS2ΔCUE. Cell lysates were immunoblotted with anti-PS1 NTF, anti-PS1 CTF or anti PS2 CTF antibodies, respectively. As anticipated, mutagenesis of PS1 catalytic sites (Asp257/Asp385) or deletion of PS1 or PS2 CUE domains inhibited the endoproteolysis of PS1 and PS2, and abolished the formation of PS1/PS2 endoproteolytic NTF/CTF fragments **(Figure 3.9 and Figure 3.10)**. However, mutagenesis of the conserved single residues within PS1 CUE domain had no effect on PS1 endoproteolysis.

3.2.2 TRAF6 enhances immunodetection of PS1/PS2 CUE domain deletion mutants

We have already demonstrated that TRAF6 enhances PS1 immunodetection and that PS1/PS2 CUE domain deletion mutants are not able to undergo presenilin endoproteolysis into NTF/CTF fragments. We next sought to examine whether the PS1ΔCUE and PS2ΔCUE mutants, which lack the CUE domain and the ability to undergo endoproteolysis, are still stabilized by TRAF6. HEK293T cells were transfected with PS1ΔCUE or PS2ΔCUE, and co-transfected with increasing amounts of FLAG-TRAF6. Wild type PS1 and PS2 were also transfected with increasing amounts of TRAF6 as positive controls. Cell lysates were examined by Western blotting with antibodies to PS1 NTF and PS2 CTF. Consistent with previous data, co-expression with increasing concentration of TRAF6 resulted in increased wild type PS1 and PS2 full length and fragment immunodetection. However, increased levels of full length PS1ΔCUE and PS2ΔCUE were also observed **(Figure 3.11A and B)**, suggesting that the enhancing effects of TRAF6 on presenilin immunodetection are independent of the putative CUE domain in PS1 and PS2 or the endoproteolysis of

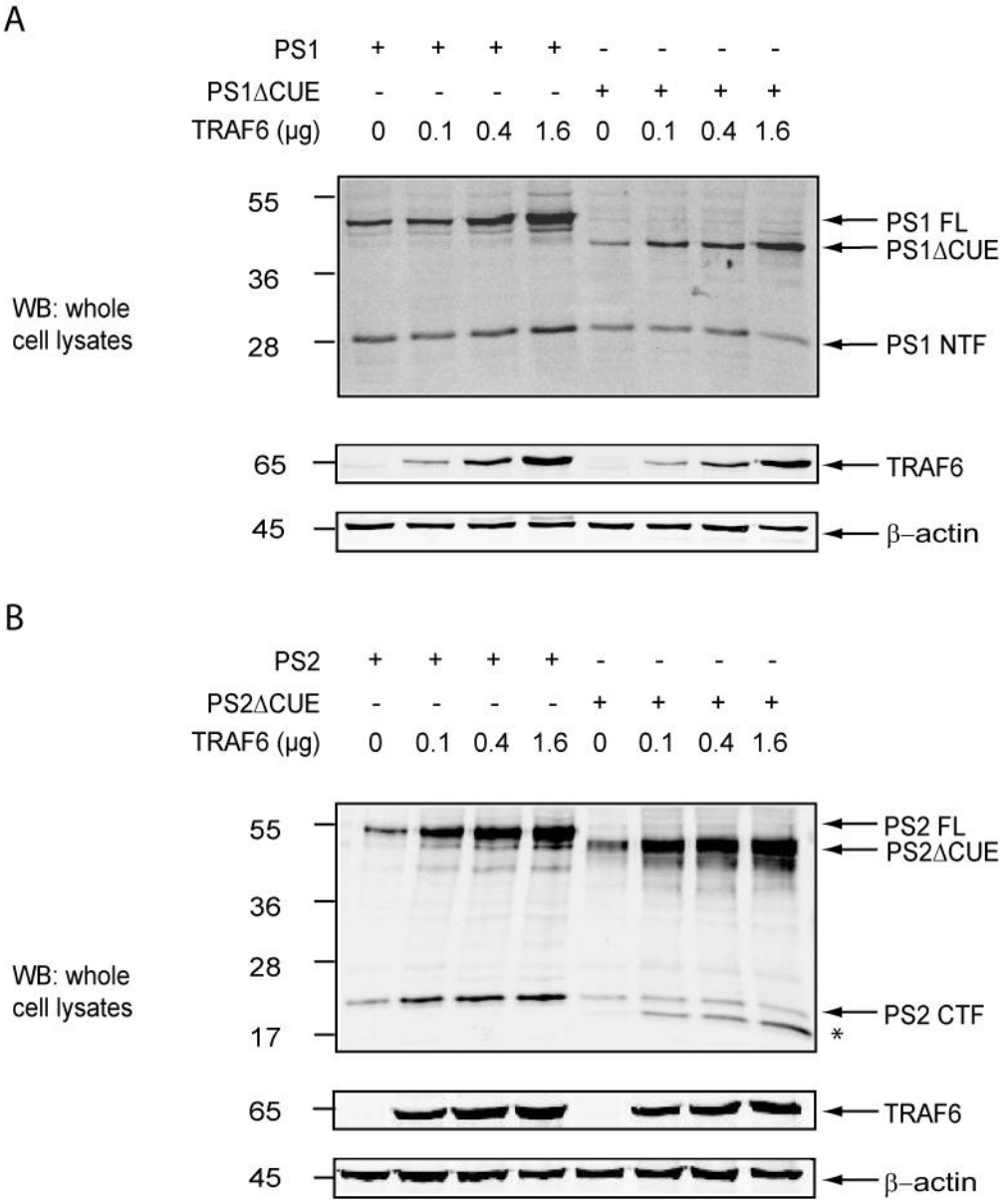

Figure 3.11 TRAF6 enhances immunodetection of PS1ΔCUE and PS2ΔCUE. HEK293T cells were transfected with expression constructs encoding wild type PS1 and PS1ΔCUE **(A)** or wild type PS2 and PS2ΔCUE **(B)** and co-transfected with increasing amounts of FLAG-TRAF6. Cell lysates were analyzed by Western blot with anti-PS1 NTF or anti-PS2 CTF, anti-FLAG and anti-β-actin antibodies. Asterisk indicates a series of bands emerging with co-expression of PS1ΔCUE and TRAF6. Data are representative of one typical experiment repeated three times.

presenilins. PS1 and PS2 fragments observed with ΔCUE mutants are endogenous wild-type presenilin fragments in HEK293T cells and are not affected by overexpression of TRAF6, suggesting that TRAF6 does not regulate presenilin fragments. The observed increases in presenilin fragments arising from overexpression of TRAF6 arise as a consequence of the increased endoproteolytic full-length presenilins. Interestingly, a series of bands which could be caspase-cleaved PS2CTF were detected in cells transfected with PS2ΔCUE and increasing amounts of TRAF6 **(Figure 3.11B lane 5-8 asterisk)**.

3.2.3 TRAF6 enhances PS1 immunodetection, independent of its CUE domain and γ-secretase complex catalytic sites

Having shown that PS1ΔCUE and PS2ΔCUE immunodetection is enhanced by TRAF6, we hypothesised that single point mutants in the CUE domain and biologically inactive aspartate mutant might still be stabilized by TRAF6. To test this hypothesis, HEK293T cells were transfected with wild type PS1, PS1D257A/D385A, PS1ΔCUE, PS1S310A, PS1V309A, PS1F283A or PS1L271A, and co-transfected with or without TRAF6. Cell lysates were examined by Western blotting with an anti-PS1 NTF antibody. Again, detection of full length wild type PS1 and mutants was enhanced by co-expression of TRAF6 **(Figure 3.12)**. Following densitometry analysis, levels of full length PS1 and its mutants are increased by TRAF6 by 2-6 folds. The especially high increased levels of full length PS1ΔCUE and PS1V309A are ascribed to their low expression levels when expressed on their own. In parallel to the study presented in this thesis, our group has also shown that PS1 and PS2 binds to K63-linked polyubiquitin chains through the conserved CUE domain and that deletion of the

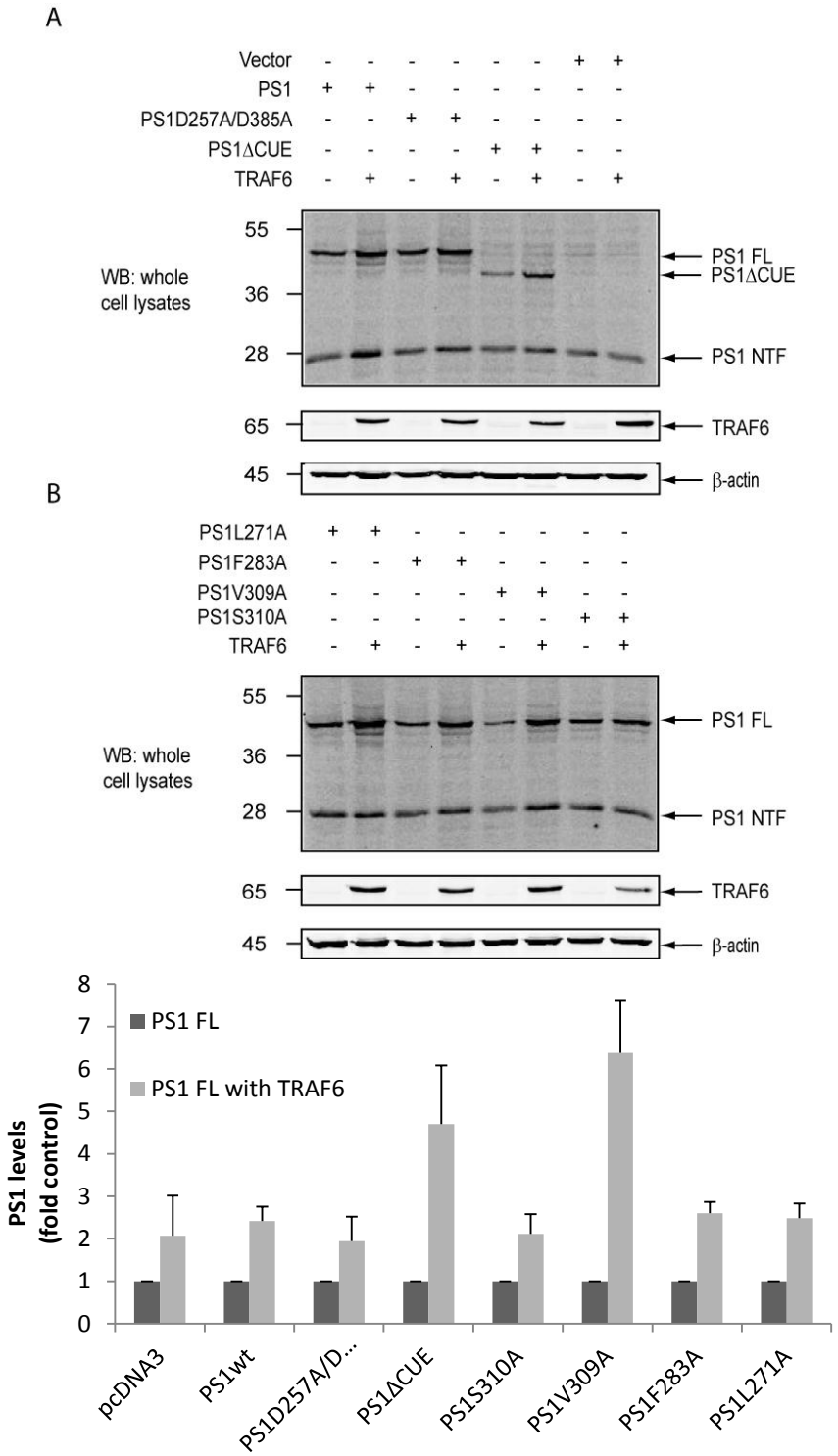

Figure 3.12 TRAF6 enhances PS1 immunoreactivity independent of its CUE domain and γ-secretase catalytic sites. (A) and **(B)**, HEK293T cells were transfected with wild type PS1, PS1D257A/D385A, PS1ΔCUE, PS1S310A, PS1V309A, PS1F283A or PS1L271A and co-transfected with TRAF6 as indicated. Cell lysates were analyzed by Western Blot with anti-PS1 NTF, anti-FLAG and anti-β-actin antibodies. **(C)** PS1 full length (FL) and fragment levels were measured by densitometry. Data shown are mean + SEM of triplicate samples.

CUE domain or mutation of the valine residue within the CUE domain abolishes PS1 binding to polyubiquitin, indicating a potential role of this newly identified CUE domain of PS1 and PS2 in presenilin functionality. As PS1D257A/D385A full length is still enhanced by TRAF6, we can conclude that neither the effect of TRAF6 on PS1 depends on the integrity of the CUE domain, nor on PS1-dependent γ-secretase activity.

3.2.4 Deletion of the putative CUE domain has no effect on the turnover of PS1

As we have shown that the putative PS1 CUE domain deletion mutant is unable to undergo endoproteolysis (**Figure 3.9**), we were interested in whether full length PS1ΔCUE has distinct turnover comparing to wild type PS1. To test this we performed a comparative analysis of the half-life of PS1 and PS1ΔCUE proteins. HEK293T cells were transfected with PS1 or PS1ΔCUE. Twenty-four hours post-transfection, cell cultures were treated with cycloheximide (20μg/ml) for increasing times (0, 1, 2, 4, 8, 12 and 24 hours). Cell lysates were then analysed by Western blotting with anti-PS1 CTF antibody **(Figure 3.13)**. Similar declining trends of wild type PS1 and PS1ΔCUE were observed indicating that deletion of the CUE domain does not affect the turnover of PS1. Although deficient in endoproteolysis, PS1ΔCUE does not show any accumulation comparing to the WT PS1, revealing an effective mechanism regulating full-length presenilin levels in which TRAF6 is very possibly involved.

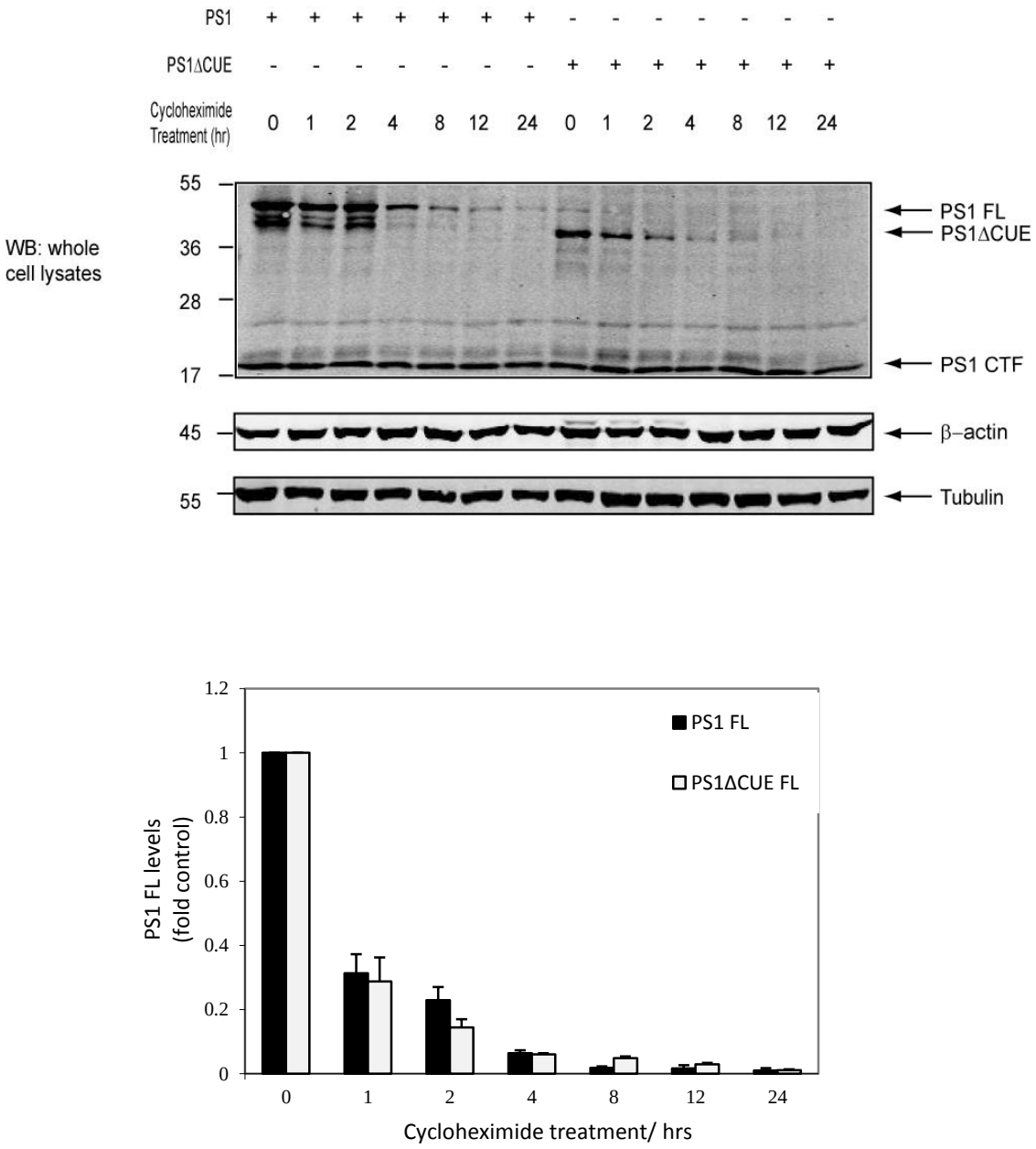

Figure 3.13 Deletion of the putative CUE domain has no effect on the turnover of PS1. HEK293T cells were transiently transfected with PS1 or PS1ΔCUE construct. Twenty-four hours after transfection, cell cultures were incubated with cycloheximide (20μg/ml) for 0, 1, 2, 4, 8, 12 and 24 hours respectively. Cell lysates were analyzed for PS1 immunodetection by immunoblotting with anti-PS1 CTF antibody. β-actin and β-tubulin levels were measured as loading control. Densitometry was used to measure levels of PS1 full length. Bars presented are the mean + SEM of triplicate samples. No statistical significance was observed between these two groups ($p>0.1$).

3.3 Presenilins are novel substrates of TRAF6-mediated ubiquitination

3.3.1 TRAF6 ubiquitinates PS1 and PS1ΔCUE via Lys63-linked polyubiquitination

Alteration in the ubiquitination status of PS1 has been shown to prevent PS1 proteasomal degradation and increase PS1 stability (Aoyagi et al., 2010). Given that TRAF6 is an E3 ubiquitin ligase and that TRAF6 alters stability of PS1, we hypothesized that PS1 may be a novel ubiquitination substrate of TRAF6. To test whether PS1 is ubiquitinated by TRAF6 and what the ubiquitination type that TRAF6 induces is, HEK293T cells were transiently transfected with wild type PS1 or PS1ΔCUE and co-transfected with TRAF6 or TRAF6-DN and co-transfected with HA-tagged ubiquitin (Ub) or the ubiquitin mutant, UbK63R, that is unable to form Lys63-linked polyubiquitination chain. Thirty-six hours post-transfection cells were harvested under stringent 1% SDS lysis condition and PS1 was immunoprecipitated with anti-PS1 NTF antibody. Ubiquitination of PS1 was detected via Western blotting with anti-HA antibody **(Figure 3.14)**. A slight smear was detected where PS1 was co-expressed with ubiquitin which indicates the ubiquitination of PS1 **(Figure 3.14 lane 3)**. Expression of PS1 and PS1ΔCUE with TRAF6 and ubiquitin showed much stronger smears suggesting that both PS1 and PS1ΔCUE are ubiquitinated by TRAF6 **(Figure 3.14 lane 4 and 6)**. Co-expression of PS1, TRAF6 and UbK63R showed no smear when probed with anti-HA antibody, but showed strong smear when the blot was re-probed with anti-PS1 NTF antibody (**Figure 3.14 lane 7 top and middle panel)**, indicating that polyubiquitination of PS1 is formed through ubiquitin Lys63 residue

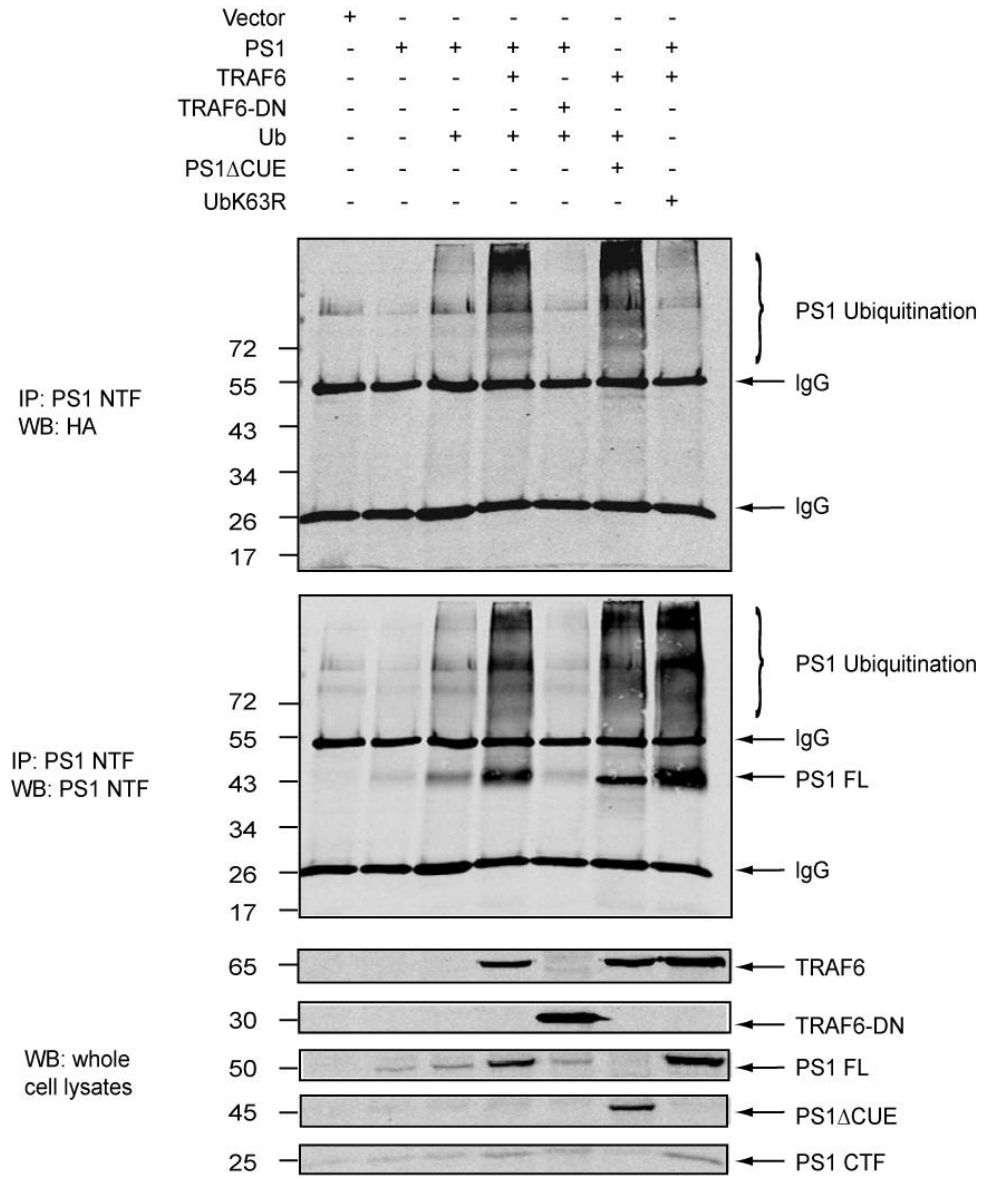

Figure 3.14 TRAF6 induces Lys63-linked polyubiquitination of PS1. HEK293T cells were transfected with wild type PS1 or PS1ΔCUE and co-transfected with TRAF6 or TRAF6-DN and co-transfected with HA tagged ubiquitin (Ub) or UbK63R, as indicated. Under stringent SDS-denaturing immunoprecipitation condition, cell lysates were prepared and immunoprecipitated with anti-PS1 NTF antibody thirty-six hours after transfection. Precipitated PS1 and ubiquitination of PS1 were detected by immunoblotting with anti-PS1 NTF and anti-HA epitope antibodies. Western blot of whole cell lysates confirms equal expression of all constructs. Date shown is representative of a typical experiment which was repeated at least three times.

and TRAF6 could induce polyubiquitination via endogenous wild-type ubiquitin in the present of UbK63R mutant. Immunoglobulin G (IgG) of PS1 NTF antibody was also detected as marked in the figure.

3.3.2 Knock-out of TRAF6 decreases ubiquitination and full-length level of PS1

After showing that TRAF6 induces ubiquitination of PS1, we assumed that knock-out of TRAF6 should have the opposite effect on PS1. To verify the hypothesis, PS1 was transfected alone or co-transfected with HA-Ub in control MEF cells and $TRAF6^{-/-}$ MEF cells. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for PS1 and Western blot for HA-Ub. Ubiquitination of PS1 was observed when PS1 and HA-Ub were co-expressed in control MEF cells; however in $TRAF6^{-/-}$ MEF cells this ubiquitination was diminished (**Figure 3.15**) suggesting that TRAF6 is required for the ubiquitination of PS1. Moreover, when checking PS1 expressions by Western blot, we showed that in the absence of TRAF6, increasing overall cellular ubiquitination by over-expressing HA-Ub decreased full-length level of PS1 (**Figure 3.15 lower panel lane 6**), suggesting that TRAF6-mediated ubiquitination positively regulates the stability of full-length PS1 and some unknown E3 ligases may play the opposite role.

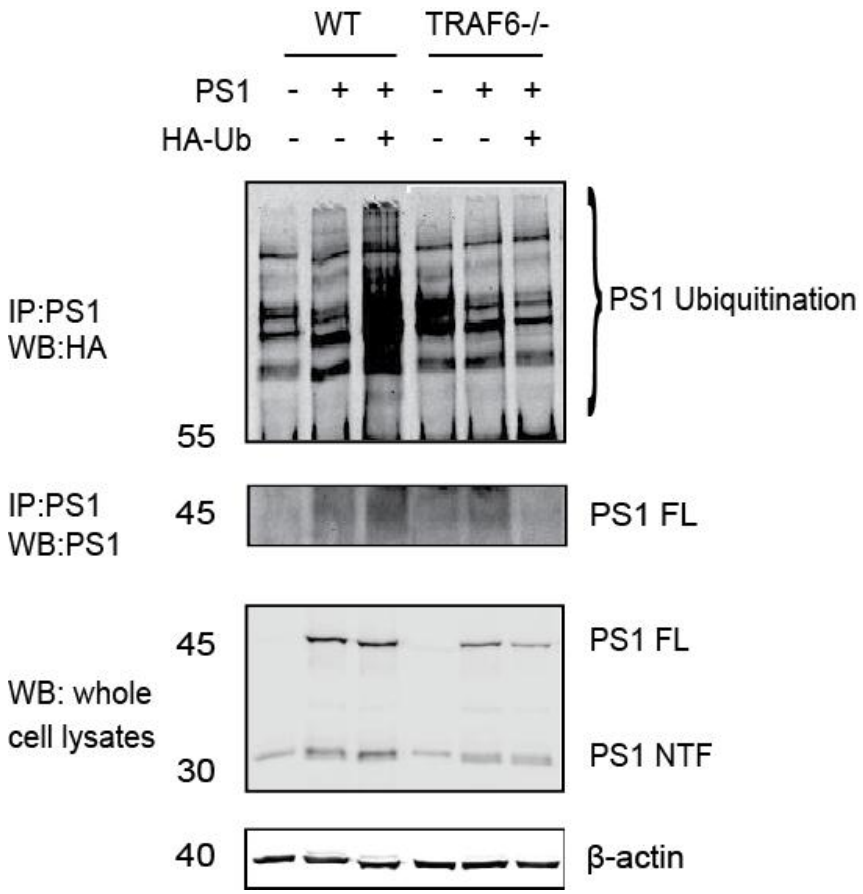

Figure 3.15 Knock-out of TRAF6 decreases ubiquitination and full-length level of PS1. Wild-type MEF cells and TRAF6 knock-out MEF cells were transfected with PS1 or co-transfected with HA-Ub. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for PS1 and Western blot for HA-Ub. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of PS1. Precipitated PS1 was detected by anti-PS1 NTF antibody. Western blot of whole cell lysates confirms expressions of all proteins.

3.3.3 Substrate specificity of PS1 ubiquitination comparing different E3 ligases.

Having shown that PS1 is ubiquitinated by TRAF6, we next investigated the specificity of PS1 ubiquitination by testing other TRAF family members and E3 ligases. We introduced two known E3 ligases: PDLIM2 (PDZ and LIM domain 2) and βTRCP (beta-transducin repeat-containing homologue protein) (Tanaka et al., 2007; Limon-Mortes et al., 2008). HEK293T cells were transiently transfected with wild type PS1 and co-transfected with HA-Ub and co-transfected with TRAF6, TRAF6C70A, TRAF2, TRAF5, PDLIM2 or βTRCP. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation and Western blot. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of PS1. While TRAF2 did induce a detectable increase in PS1 ubiquitination, co-expression of PS1, HA-Ub and TRAF6 or TRAF5 showed the strongest increase in PS1 ubiquitination (**Figure 3.16 lane 3, 5 and 6**). Importantly, co-expression of PS1, HA-Ub and the TRAF6 E3 ligase activity deficient mutant, TRAF6C70A, showed minimal increase in PS1 ubiquitination over background (co-expression of PS1 and HA-Ub) (**Figure 3.16 lane 4**), demonstrating that the observed increase in PS1 ubiquitination was TRAF-dependent. Furthermore, co-expression of βTRCP or PDLIM2 with PS1 and HA-Ub did not increase the ubiquitination of PS1 (**Figure 3.16 lane 7 and 8**), revealing that PS1 was not ubiquitinated by these two E3 ligases. Collectively, our results suggest that ubiquitination of PS1 is specific to TRAF family E3 ligases.

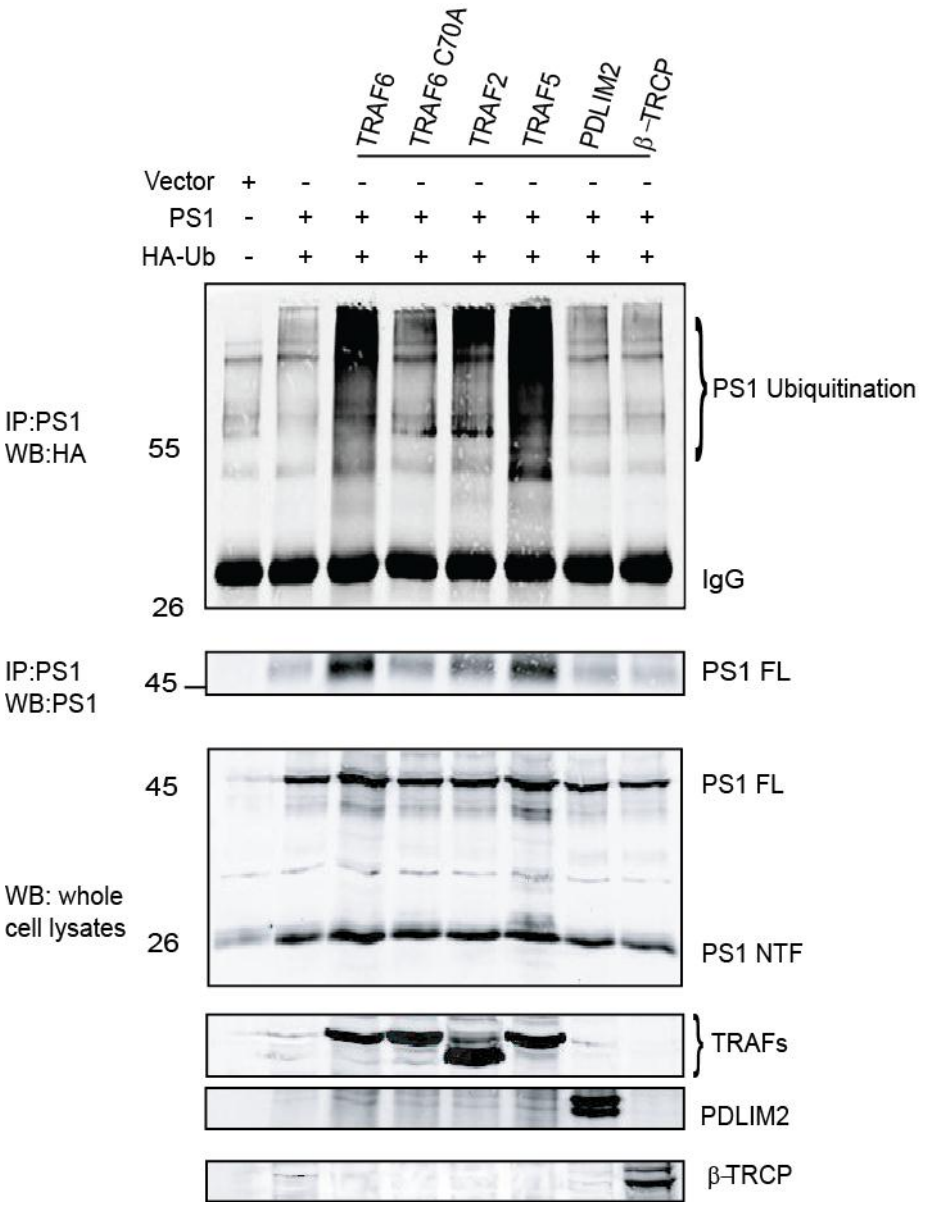

Figure 3.16 PS1 is preferentially ubiquitinated by TRAF family E3 ligases. HEK293T cells were transiently transfected with wild type PS1 and HA-Ub and co-transfected with TRAF6, TRAF6C70A, TRAF2, TRAF5, PDLIM2 or βTRCP. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation and Western blot. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of PS1. Precipitated PS1 was detected by anti-PS1 NTF antibody. Western blot of whole cell lysates confirms expressions of all proteins. Experiment was repeated more than three times showing the same result.

3.3.4 Only full-length PS1 is ubiquitinated by TRAF6, but not its fragments.

Having shown PS1 as a substrate of TRAF6-mediated ubiquitination, we next investigated wheter full-length PS1 or either of its proteolytic fragments PS1-NTF or PS1-CTF are ubiquitinated by TRAF6. HEK293T cells were transiently transfected with full-length PS1 or PS1 NTF or PS1 CTF truncated mutants and co-transfected with HA-Ub and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation and Western blot. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of full-length PS1 (**Figure 3.17**). No smear or obvious alteration was detected in the lower molecular panel when probing for HA-ubiquitin suggesting that PS1-NTF and PS1-CTF truncated fragments are not ubiquitinated by TRAF6. Western blot analysis of the whole cell lysates showed that only the levels of full-length PS1 were dramatically increased by co-overexpression of ubiquitin and TRAF6, but not either of the NTF or CTF fragments. Collectively, our results suggest that TRAF6 regulates only the full-length PS1, but not the PS1 fragments. As endoproteolysis of presenilin is considered to take place during its transport from ER to Golgi apparatus (Spasic et al., 2006b), regulation of the presenilins by TRAF6 should be revealed as an early cellular modulating event. In addition, whether regulation of TRAF6 has any effect on full-length presenilin functions should be examined.

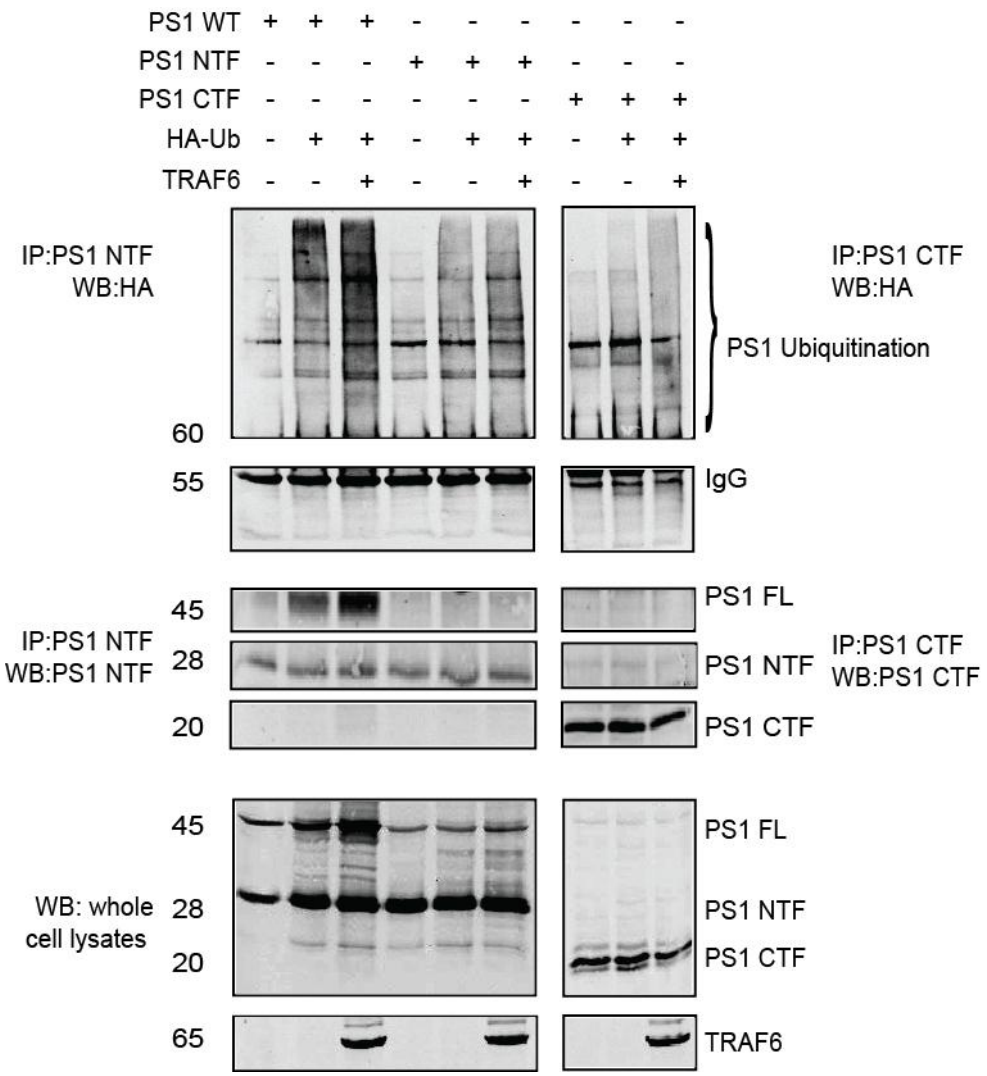

Figure 3.17 Only full-length PS1 is ubiquitinated by TRAF6, but not its fragments. HEK293T cells were transiently transfected with wild type PS1, PS1 NTF or PS1 CTF and co-transfected with HA-Ub and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then were subjected to immunoprecipitation for PS1 full-length, PS1 NTF or PS1 CTF. Ubiquitination status of immunoprecipitated PS1 full-length or fragments were revealed by Western blot analysis using anti-HA antibody. Western blot analysis of the whole cell lysates showed the levels of overexpressed PS1 full-length and its fragments.

3.3.5 TRAF6 increases the level of full-length APP as well as the levels of its intracellular and extracellular fragments

Presenilins are the catalytic core of γ-secretase complex which facilitates the γ-secretase dependent cleavage of APP (Citron et al., 1997). Abnormal production of the Aβ peptides is considered as the key pathogenesis of AD. As TRAF6 was shown to regulate the cellular levels of presenilin, we next investigated the effect of TRAF6 on APP and its cleavage. HEK293T cells are transfected with APP Swedish mutant (APP SW) alone or co-transfected with TRAF6. Twelve hours after transfection, selected cell cultures were treated with the γ-secretase inhibitor, Compound E (50nM), as indicated. Cells were harvested 24 hours after transfection. Lysates were subjected to Western blot analysis and probed with an anti-APP C-terminus antibody. Overexpression of TRAF6 increases the detectable level of full-length APP as well as the APP cleavage products, APP C99, APP C83 and APP AICD (**Figure 3.18A**). In parallel, culture medium was collected from cells expressing APP SW or co-expressing APP SW and TRAF6 and was subjected to ELISA for the analysis of Amyloid peptides, Aβ40 and Aβ42. Consistent to the Western blot result where over-expression of TRAF6 increases the detectable levels of APP and its proteolytic fragments, levels of soluble Aβ40 and Aβ42 were also increased (**Figure 3.18B**). As TRAF6 increases the overall levels of APP and its proteolytic fragments, using this experimental approach we are unable to conclude whether or not TRAF6 directly affects γ-secretase activity.

3.3.6 Over-expression of TRAF6 does not affect γ-secretase activity

Having shown the association between TRAF6 and the increased levels of presenilins and APP, we wanted to determine whether or not the observed effect of TRAF6 is

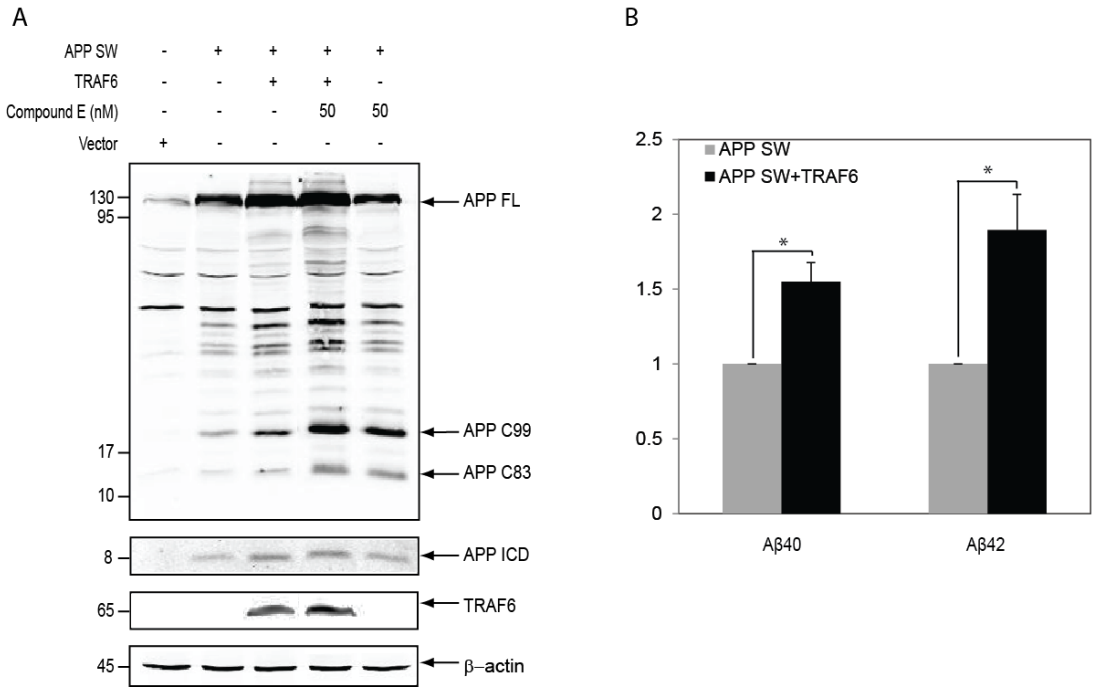

Figure 3.18 TRAF6 increases the level of full-length APP as well as the levels of its intracellular and extracellular fragments. (A) HEK293T cells were transfected with APP SW and co-transfected with FLAG-TRAF6. 12 hours after transfection, selected cell cultures were treated with Compound E (50nM) as indicated. Cells were harvested 24 hours after transfection. Lysates were subjected to Western Blots and probed with anti-APP C-terminal, anti-FLAG and anti-β-actin antibodies. **(B)** Culture medium **(A lane 2 and 3)** was collected before harvest and subjected to ELISA for Aβ40 and Aβ42. Data is presented by mean + SEM of three independent experiments. Difference observed between these two groups was significant ($p<0.05$).

specific for selected target proteins or a general feature on cellular proteins. Because TRAF6 associates with both PS1 and PS2 (Figure 3.1) and preferentially alters detectable levels of full-length PS1 and PS2 but not γ-secretase protease components, PS1-NTF and PS1-CTF (Figure 3.17), we decided to determine if TRAF6 has any effect on the other γ-secretase protease components, Nicastrin, Aph-1 or Pen-2. Cells over-expressing empty vector or TRAF6 were lysed and TRAF6 was immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were then analysed for co-immunoprecipitation of Aph-1, Nicastrin, Pen-2 and/or PS1 by Western blotting. Interestingly, only PS1 coprecipitated with TRAF6 (**Figure 3.19A left panel**). Whole cell lysates were also analysed by Western blotting for the levels of the γ-secretase components and then were quantitated by densitometry. Results suggest that TRAF6 only increased detectable levels of full-length PS1, but not any of the other γ-secretase components (**Figure 3.19A middle and right panel**).

In previous reports, we have linked the over-expression of TRAF6 to the cellular levels of two γ-secretase substrates, IL1-R1 and $p75^{NTR}$ (Powell et al., 2009; Twomey et al., 2009). We also detected linkage between over-expression of TRAF6 and the levels of APP and its γ-secretase proteolytic products (**Figure 3.18**). Next, we decided to measure the effect of TRAF6 on γ-secretase activity by studying the cleavage of a truncated mutant of APP, APP CT100, which is the natural substrate for γ-secretase proteolysis and is directly processed by γ-secretase. In HEK293T cells, APP CT100 was transiently transfected alone or co-transfected with TRAF6, and cell lysates were subjected to Western blot analysis and probe with an anti-APP C-terminus antibody.

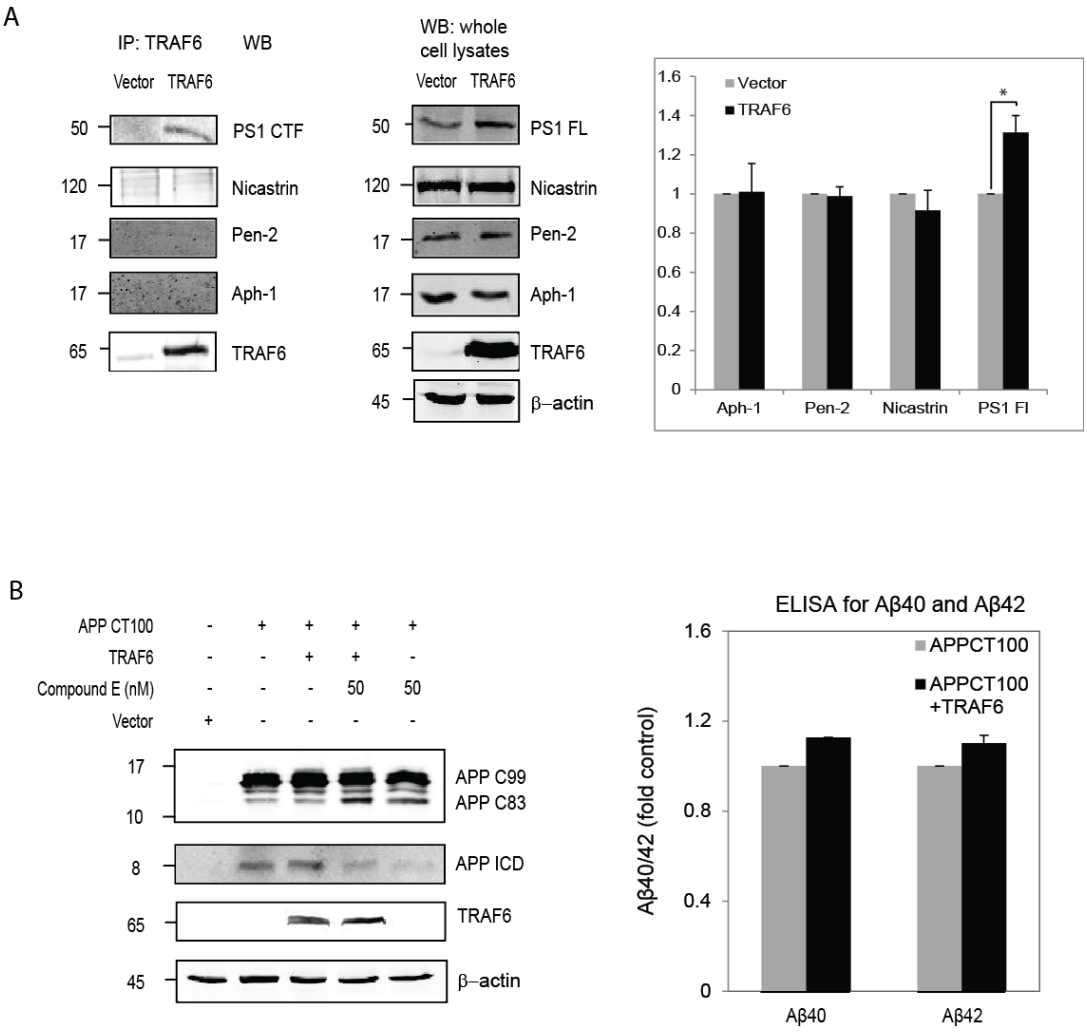

Figure 3.19 Over-expression of TRAF6 does not affect γ-secretase activity. (A) HEK293T cells were transfected with empty vector or FLAG-TRAF6 for 36 hours. Cell lysates were then subjected to immunoprecipitation for TRAF6 and Western blot for the γ-secretase components as indicated. Cell lysates were also analysed by Western Blot with anti-Aph-1, anti-Nicastrin, anti-Pen-2, anti-PS1 NTF, anti-FLAG and anti-β-actin antibodies. Protein levels were measured by densitometry. Results are presented by mean + SEM from three independent experiments. Only difference of PS1 full-length levels between these two groups are statistically significant ($p<0.05$). **(B)** HEK293T cells were transfected with APP CT100 and co-transfected with FLAG-TRAF6. 12 hours after transfection, selected cell cultures were treated with Compound E (50nM) as indicated. Cells were harvested 24 hours after transfection. Lysates were subjected to Western Blots and probed with anti-APP C-terminal, anti-FLAG and anti-β-actin antibodies. Culture medium **(B lane 2 and 3)** was collected before harvest and subjected to ELISA for Aβ40 and Aβ42. Data is presented by mean + SEM of three independent experiments. No significant difference was observed between these two groups.

Our result showed similar levels of the APP CTD and ICD with or without over-expression of TRAF6 which indicates that TRAF6 does not induce any major change on the cleavage of APP CT100, and therefore on γ-secretase activity (**Figure 3.19 B lane 2 and 3**). Treatment of parallel cultures with the γ-secretase inhibitor, compound E, confirmed that APP C100 derived proteolytic fragments observed are products of γ-secretase cleavage (**Figure 3.19 B lane 4 and 5**). To further confirm the result with a more sensitive and quantifiable assay, cell culture medium was collected and levels of Aβ40 and Aβ42 peptides were measured using an ELISA assay (**Figure 3.19B right panel**). Considering the effect of TRAF6 on endogenous APP, TRAF6 does not induce a significant change in γ-secretase activity or the cleavage of APP CT100, suggesting that γ-secretase activity is not altered by over-expression of TRAF6.

3.3.7 Knock-out of presenilins or TRAF6 attenuates ER calcium signalling

The observation that TRAF6 regulates full-length presenilins but not the γ-secretase activity lead us to focus on the known biological function of presenilin holoprotein, independent of presinilin function in γ-secretase protease complexes. Recent studies have revealed that presenilins function as the passive ER Ca^{2+} leak channels and FAD mutants cause deranged Ca^{2+} signalling and cannot rescue the deregulated ER Ca^{2+} leak activity in presenilin knock-out MEFs (Tu et al., 2006; Nelson et al., 2007; Nelson et al., 2010). To determine if TRAF6-mediated ubiquitination of presenilins, regulated presenilins function as an ER Ca^{2+} leak channel, firstly, we compared the ER Ca^{2+} leak activity in control MEF cells and presenilin double knock-out (DKO) MEF cells. MEF cells were cultured to grow to about 50% confluency. Prior to experimentation cells

were washed twice with 1ml of KHB and then were loaded with 2 μM fura-2-acetoxymethyl ester and incubated for 30 min at 37°C, as described in materials & methods. Cells were then washed twice with 1ml of Ca^{2+}-free KHB and loaded with 5 μl of 1mM Ionomycin to induce complete depletion of the ER calcium store which could be measured to indicate the size of the ER Ca^{2+} pool. Alterations in the cytosolic Ca^{2+} levels were detected by exciting the fura-2 loaded cells intermittently by 340 and 380 nm UV light. After recording for 10 min, ratio images were generated and the perimeter of each cell was defined as a region of interest and the mean fura-2 ratio from within this region against time was exported to Microsoft Excel 2003 for further analyses. **Figure 3.20A** shows an example of the ratio/time curves of both control MEFs and presenilin DKO MEFs reflecting the size of the ER Ca^{2+} store and the ability of presenilins as passive ER Ca^{2+} leak channels. Collectively, by showing that presenilin DKO MEF cells exhibited higher level of Ca^{2+} exposure, we reproduced the experiment showing attenuated passive ER Ca^{2+} leaking function in the presenilin DKO MEF cells compared to control MEF cells (**Figure 3.20B**) (Tu et al., 2006). Furthermore, to determine the role of TRAF6 in ER Ca^{2+} signalling function, we compared the ER Ca^{2+} pool size of both control MEF cells and TRAF6 knock-out MEF cells. Similar to the observation in presenilin DKO MEF cells, TRAF6 knock-out MEF cells also showed enlarged ER Ca^{2+} pool (**Figure 3.20C**), suggesting that knock-out of presenilins or knock-out of TRAF6 both result in deficiency in ER Ca^{2+} signalling.

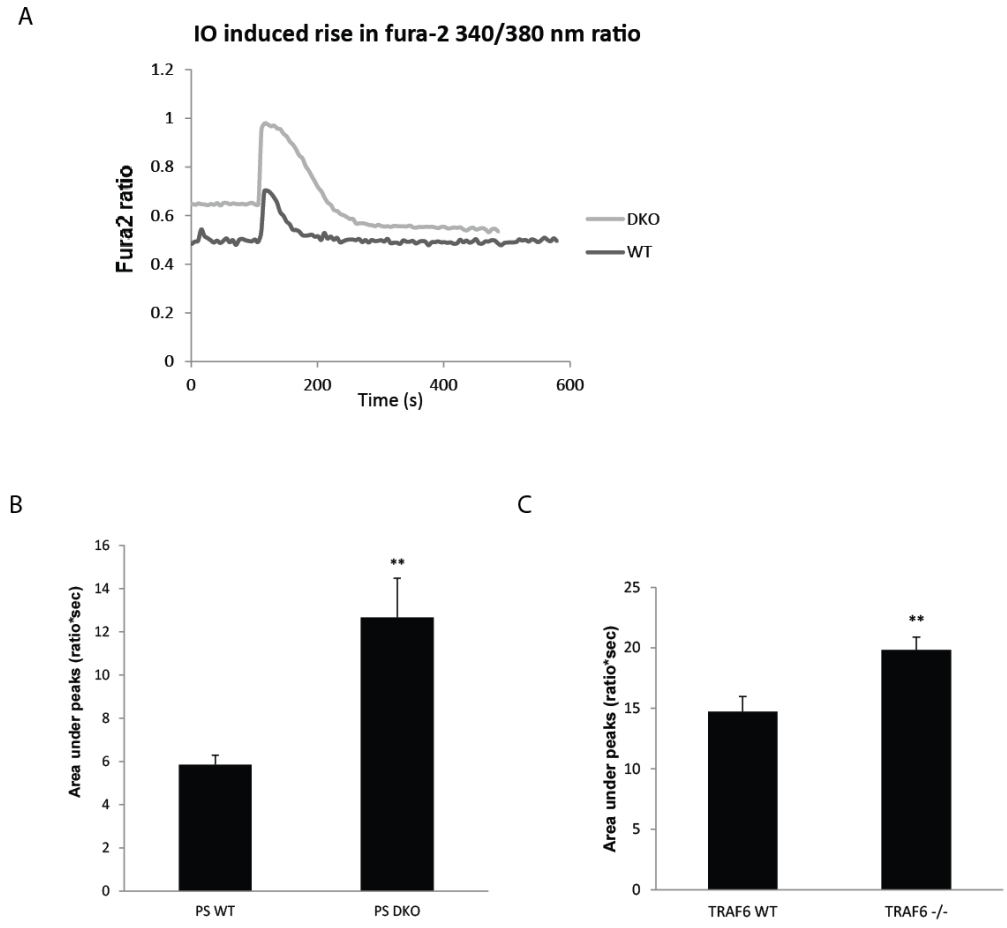

Figure 3.20 Knock-out of presenilins or TRAF6 attenuates ER calcium signalling. wild type MEF (PS WT) cells and presenilin double knock-out (PS DKO) MEF cells or wild type MEF (TRAF6 WT) cells and TRAF6 knock-out MEF (TRAF6 -/-)cells were cultured to grow to about 50% confluency. Prior to experimentation cells were washed twice with 1ml of KHB and then were loaded with 2 µM fura-2-acetoxymethyl ester and incubated for 30 min at 37°C. Cells were then washed twice with 1ml of Ca^{2+}-free KHB and loaded with 5 µl of 1mM Ionomycin to induce ER calcium discharge. Alterations in the cytosolic Ca^{2+} levels were detected by exciting the fura-2 loaded cells intermittently by 340 and 380 nm UV light. After recording for 10 min, ratio images were generated and the perimeter of each cell was defined as a region of interest and the mean fura-2 ratio from within this region against time was exported to Microsoft Excel 2003 for further analyses. **(A)** Examples shown are the Ionomycin-induced Ca^{2+} signal curves in PS WT cells (dark line) and PS DKO cells (light line). **(B)** The average intensity of cytosolic Ca^{2+} exposure for both PS WT cells and PS DKO cells are shown as the mean + SEM from 21 cells measured from each cell line. **(C)** Ionomycin induced cytosolic Ca^{2+} concentration increase and restoration were recorded for both TRAF6 WT MEF cells and TRAF6 -/- MEF cells and shown as mean + SEM measured from 17 and 27 cells respectively.

3.3.8 TRAF6-mediated *in vitro* ubiquitination of PS1

Different approaches have been carried out to optimize the *in vitro* ubiquitination assay for PS1. Because previous attempts to produce and purify recombinant PS1 from *E.coli* cells have failed, we developed an *in vitro* ubiquitination assay utilizing; recombinant TRAF6 from BL21 *E.coli* cells, immunopurified PS1 from HEK293T cells, and an *in vitro* ubiquitination kit from Biomol (including human recombinant E1 and Ubc13/Mms2 E2 recombinant enzymes, recombinant ubiquitin and ATP). However, recombinant TRAF6 from bacteria cells did not possess measurable E3 ligase activity. Therefore, we resigned to using immunopurified TRAF6 from HEK293T cells over-expressing FLAG-tagged TRAF6 extracted by recombinant 3XFLAG peptides which competes for the FLAG affinity agarose with FLAG-TRAF6. In vitro ubiquitination assays were performed by incubating immobilized PS1 conjugated to protein-G beads with recombinant E1 and E2 enzymes, recombinant ubiquitin, ATP and immunopurified TRAF6 at 37^{O}C for 1 hour. Next, the PS1 conjugated beads were washed and then boiled in sample loading buffer and subjected to Western blot analysis with an anti-ubiquitin antibody. We successfully demonstrated that TRAF6 ubiquitinated PS1 under these experimental conditions (**Figure 3.21A**). However, TRAF6 co-immunoprecipitated with immobilized PS1 during the incubation and was also observed on the Western blot (**Figure 3.21A lowest panel**).

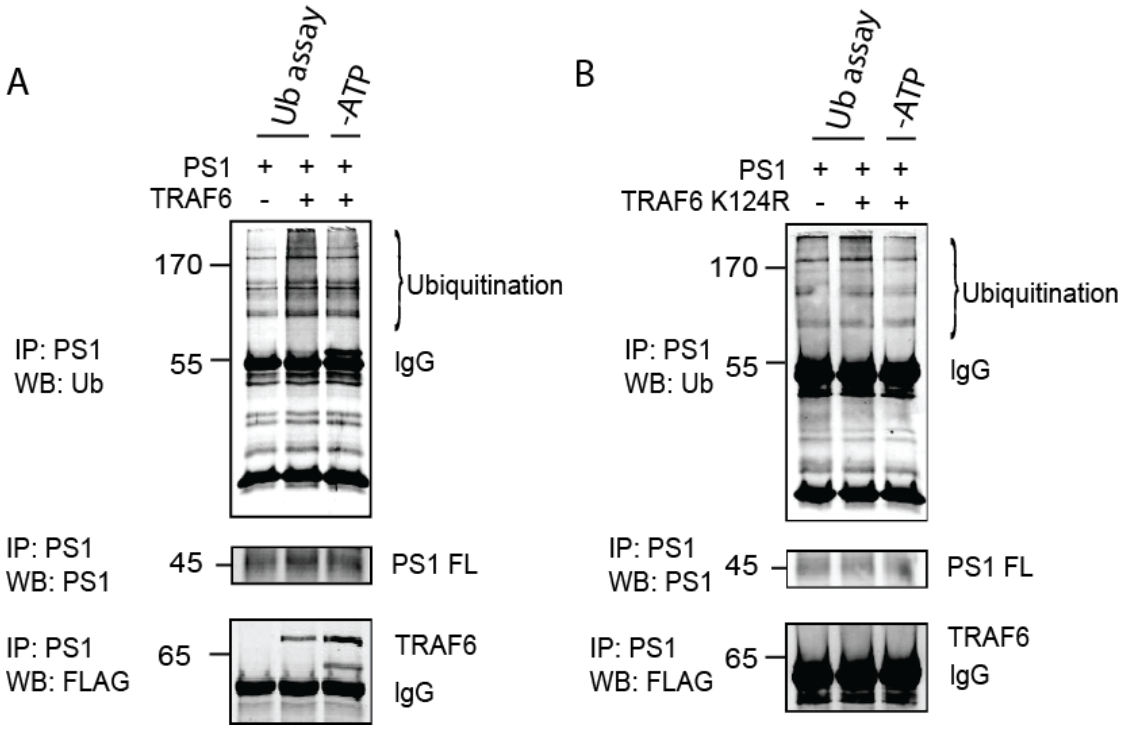

Figure 3.21 TRAF6-mediated *in vitro* ubiquitination of PS1. HEK293T cells were transfected with PS1 or FLAG-TRAF6 (**A**) or FLAG-TRAF6 K124R (**B**) respectively. 36 hours after transfection, cells were harvested and the lysates were subjected to immunoprecipitation for PS1 and FLAG-TRAF6 or FLAG-TRAF6 K124R respectively. TRAF6 conjugated FLAG affinity agarose was washed and incubated with 50 µl of 100µg/ml 3xFLAG peptide solution for 30 mins. PS1 conjugated protein-G beads were also washed and incubated with 2µM E1, 0.5mg/ml E2, 50µM ubiquitin, 100unit/ml inorganic pyrophophatase, 50mM DTT, 0.1M Mg-ATP and 20µl of TRAF6 or TRAF6 K124R elution in 50µl reaction solution at 37 ^{o}C for 1 hour. After reaction, PS1 conjugated beads were washed again and boiled in sample loading buffer for 5 mins. Sample loading buffer was then loaded on SDS-PAGE gel. Ubiquitination status was revealed by probing with anti-ubiquitin antibody. Immunoprecipitated PS1 and FLAG-TRAF6 were confirmed by probing with anti-PS1 and anti-TRAF6 antibodies.

In vitro ubiquitination assays without ATP also showed residual ubiquitin which could be attributed to auto-ubiquitination of TRAF6 (**Figure 3.21A lane3**).We tried to avoid this problem by using the TRAF6K124R mutant instead of the wild-type TRAF6. However, TRAF6K124R mutant did not appear to exert effective E3 ligase activity in the *in vitro* ubiquitination assay (**Figure 3.21B**).

3.3.9 Identification of PS1 ubiquitination sites by site-directed mutagenesis

To further study the function of TRAF6-mediated ubiquitination of PS1, we attempted to map the sites of PS1 ubiquitination by site-directed mutagenesis. There are 16 lysine residues in the human PS1 sequence, however 3 lysine residues localise in the transmembrane domain which precludes them as potential ubiquitination sites (**Figure 3.22**). Given this, we mutated the remaining 13 lysine residues and potential ubiquitin acceptor sites by site-directed mutagenesis and tested them in the *in vivo* ubiquitination assay. HEK293T cells were transfected with PS1 or the various PS1 lysine mutants and co-transfected with HA-Ub and TRAF6, as indicated (**Figure 3.23**). Thirty-six hour after transfection, cells were harvested under SDS-denaturing condition. Cell lysates were then subjected to immunoprecipitation for PS1 and Western blotting for HA-Ub with an anti-HA antibody. Unfortunately, WT PS1 and all PS1 lysine mutants showed increased ubiquitination when they are co-overexpressed with TRAF6 (**Figure 3.23**). Full-length levels of all PS1 lysine mutant were also increased by TRAF6. No obvious difference was observed in the ubiquitination profile of PS1 and any of the PS1 lysine mutants analysed, suggesting that TRAF6-mediated ubiquitination of PS1 possibly occurs at multiple lysine residues of PS1. Mutagenesis of multiple lysine residues or a different approach are required to map the ubiquitination sites of PS1.

Presenilin-1 Homo sapiens

```
        10         20         30         40         50         60
MTELPAPLSY FQNAQMSEDN HLSNTVRSQN DNRERQEHND RRSLGHPEPL SNGRPQGNSR

        70         80         90        100        110        120
QVVEQDEEED EELTLKYGAK HVIMLFVPVT LCMVVVVATI KSVSFYTRKD GQLIYTPFTE

       130        140        150        160        170        180
DTETVGQRAL HSILNAAIMI SVIVVMTILL VVLYKYRCYK VIHAWLIISS LLLLFFFSFI

       190        200        210        220        230        240
YLGEVFKTYN VAVDYITVAL LIWNFGVVGM ISIHWKGPLR LQQAYLIMIS ALMALVFIKY

       250        260        270        280        290        300
LPEWTAWLIL AVISVYDLVA VLCPKGPLRM LVETAQERNE TLFPALIYSS TMVWLVNMAE

       310        320        330        340        350        360
GDPEAQRRVS KNSKYNAEST ERESQDTVAE NDDGGFSEEW EAQRDSHLGP HRSTPESRAA

       370        380        390        400        410        420
VQELSSSILA GEDPEERGVK LGLGDFIFYS VLVGKASATA SGDWNTTIAC FVAILIGLCL

       430        440        450        460
TLLLLAIFKK ALPALPISIT FGLVFYFATD YLVQPFMDQL AFHQFYI
```

Figure 3.22 Amino acid sequence of human PS1. 13 lysine residues are highlighted in yellow. Nine transmembrane domains are underlined respectively. The CUE domain is shown as blue letters. γ-secretase cleavage sites are lighted in red. The catalytic aspartate motifs and the important PAL motif are shown in red letters.

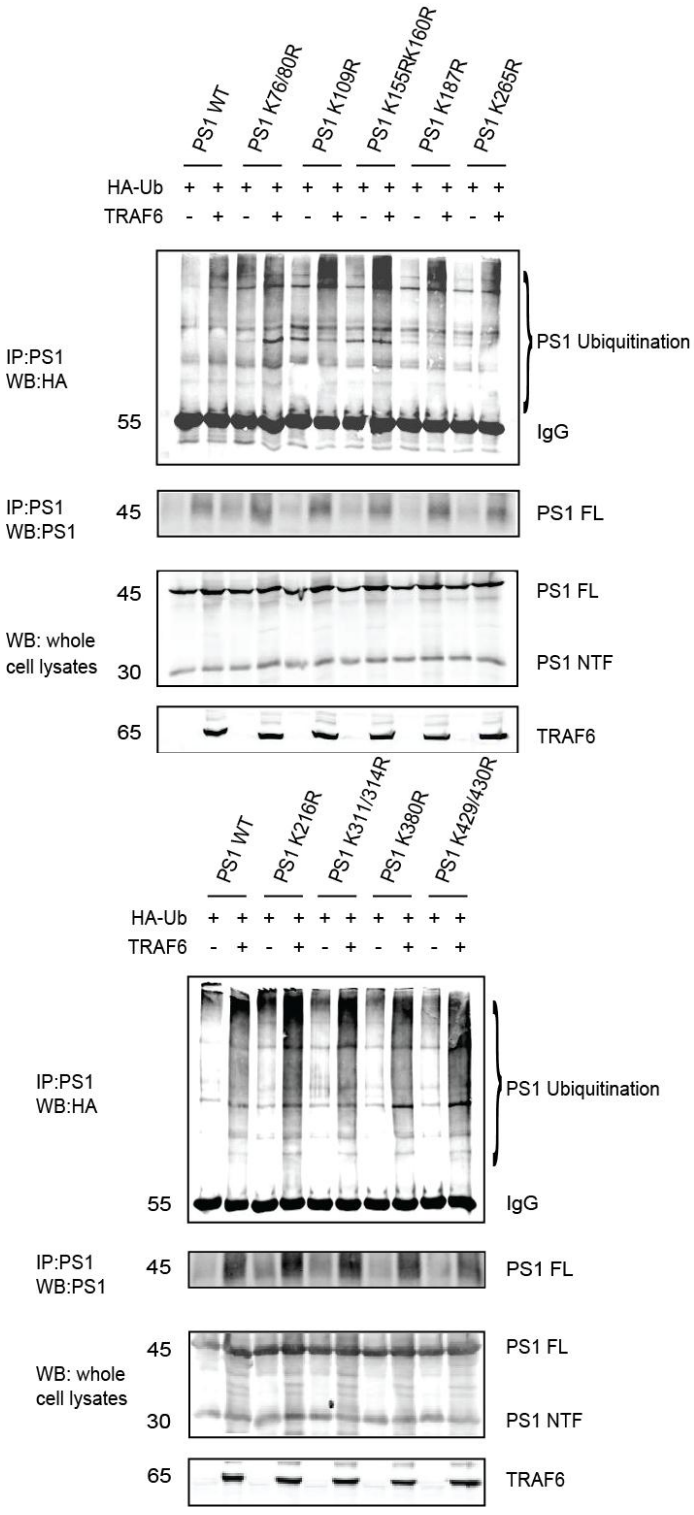

Figure 3.23 In vivo ubiquitination assays of PS1 lysine mutants. HEK293T cells were transiently transfected with wild type PS1 or PS1 lysine mutants and co-transfected with HA-Ub and TRAF6 as indicated. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation and Western blot. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of PS1. Precipitated PS1 was detected by anti-PS1 NTF antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated more than three times showing the same result.

Discussion:

The presenilin holoproteins have a relatively short half-life and the endogenous full-length proteins are maintained at relatively low levels (Ratovitski et al., 1997; Zhang et al., 1998). Full-length presenilins undergo endoproteolytic cleavage and incorporate into γ-secretase complex as NTF/CTF heterodimers during its transport from ER to Golgi apparatus (Spasic et al., 2006b). Studies have mostly focused on the functions of the NTF/CTF heterodimer as the catalytic core of γ-secretase complex and on the regulation of γ-secretase activity. However, very little is known about how the full-length presenilins are regulated in the aspects of post-translational modification, trafficking and degradation and how their endoproteolysis are modulated. Emerging studies have revealed functions of full-length presenilins including their roles in calcium signalling, β-catenin signalling, protein trafficking and apoptosis (Hass et al., 2008; Coen and Annaert, 2010; Honarnejad and Herms, 2012). In this study, we characterised and determined the relevance of the interaction between presenilins and the E3 ubiquitin ligase, TRAF6 and subsequently focused on the regulation of γ-secretase independent function of the presenilins in calcium signalling.

Our group has previously revealed an interaction between TRAF6 and presenilins which led us to the study of TRAF6-mediated regulation of presenilins (Powell et al., 2009). There are numerous reports confirming the subcellular localisation of both presenilins and TRAF6 in the plasma membrane and endosome which supports the possibility of a physical and/or functional association between presenilins and TRAF6 (Schultheiss et al., 2001; Okochi et al., 2002; Fukumori et al., 2006; Li et al., 2006a;

Zhang et al., 2006). Firstly, we confirmed the interaction between TRAF6 and both PS1 and PS2 and additionally we showed that the interaction is independent of the TRAF6 RING domain which possesses the E3 ligase activity of TRAF6 (**Figure 3.1**). We also noticed that over-expression of TRAF6 increased the detectable levels of presenilins in whole cell extracts. Then we further confirmed this effect by showing that presenilins levels are increased by TRAF6 in a dose-dependent manner, particularly the levels of full-length presenilins (**Figure 3.2**). The RING domain is reported to confer TRAF6 E3 ligase activity and is essential for TRAF6-mediate signalling pathways (Hsu et al., 1996a; Lamothe et al., 2008). In our study we investigated the relevance of TRAF6 E3 ligase activity and the effect of TRAF6 on presenilin immunodetection. Firstly we showed that TRAF6 dominant negative mutant (TRAF6DN) which lacks the critical RING domain could not increase the immunodetection of PS1 **(Figure 3.3A)**. Secondly, given that autoubiquitination of TRAF6 is reported to be independent in IL-1 and RANKL induced activation of NFκB and MAPK pathways (Walsh et al., 2008), we found that TRAF6K124R, a TRAF domain single-point mutant that is defective in TRAF6 autoubiquitination, could still increase immunodetection of the presenilins. Finally we showed that TRAF6C70A, a RING domain single-point mutant that is defective in substrate ubiquitination, failed to enhance immunodetection of the presenilins **(Figure 3.3B and C)**. Collectively, these results revealed that TRAF6 requires its E3 ubiquitin ligase activity to enhance presenilin detectable levels which means TRAF6-induced ubiquitination is involved in this regulation. Interestingly, other TRAF family members also appeared to increase the levels of PS1 and PS2 (**Figure 3.4**). To our surprise, TRAF2-DN also increases the level of presenilins indicating that TRAF2 may involve in this regulation as an adaptor

protein independent of its E3 ligase activity (**Figure 3.4**). Redundancies between TRAF family members have been reported before. For example, deficiency of both TRAF2 and TRAF6 is necessary for abrogating CD40-mediated NF-κB activation and TRAF2 and TRAF6 associate at the CD40 complex (Davies et al., 2005; Ellison et al., 2006). For TLR signalling, mutations of both TRAF2 and TRAF6 binding sites in TRIF are required to abrogate type I IFN induction (Sasai et al., 2010). Similarly, depletion of both TRAF2 and TRAF5 are required for abolishment of TNF-induced NF-κB activation (Tada et al., 2001). In our study, we showed that TRAF family members could exert similar enhancing effects on presenilin levels even in the absence of the E3 ligase activity (**Figure 3.4**).

TRAF6 has been shown to induce JNK activation and this function requires E3 ligase activity of TRAF6 (Lamothe et al., 2008). Additionally, transcription of PS1 is regulated by JNK activation (Lee and Das, 2008). To link TRAF6-increased presenilin levels to TRAF6 mediated JNK activation, we investigated PS1 protein level and mRNA level with the combination of TRAF6 over-expression and pharmacological inhibitor of JNK activity. We showed that over-expression of TRAF6 induces JNK activation, thus increasing presenilin mRNA and protein levels while inhibition of JNK activation reduces both (**Figure 3.5**). To find out if TRAF6 was involved in the regulation of PS1 post-translationally, we inhibited protein production to examine the half-life of PS1 full-length in the presence of over-expressed TRAF6.We showed that in addition to regulating PS1 transcription through JNK activation, TRAF6 also enhances the stability of PS1 and extend the half-life of PS1 full-length protein (**Figure 3.7**). Collectively, our data demonstrates that TRAF6-induced increases in the

cellular levels of presenilin should be considered as comprehensive consequences resulting from enhanced JNK activation and altered turnover of presenilin proteins.

Our group also identified a putative CUE domain in the presenilins and other team member has shown that deletion of the CUE domain abolishes K63-linked polyubiquitin chains from binding to presenilins (data not published). The CUE domain on other proteins has been shown to mediate interactions between ubiquitin and CUE domain containing proteins, which facilitates their monoubiquitination (Davies et al., 2003; Shih et al., 2003b; Chen et al., 2006). Given that monoubiquitination is a signal regulatory modification that is important in altering protein activity, location or structure (Hicke, 2001), we were interested in examining the role of the CUE domain in presenilins functions. Firstly, we showed that deletion of the presenilin CUE domain prevents presenilin endoproteolysis as the cleaving sites are contained in the CUE domain **(Figure 3.9 and 3.10)**. However, the four CUE domain single-point mutants were still able to undergo cleavage and no obvious difference was observed when compared to wild type PS1, suggesting that the single-point mutations of the critical CUE domain residues have no effect on PS1 endoproteolysis. Secondly, we showed that CUE domain deletion mutants of PS1 and PS2 were still increased in a dose-dependent manner with co-expression of TRAF6 **(Figure 3.11)**. Finally, despite that the PS1 CUE domain deletion mutant is deficient in presenilin endoproteolysis, deletion of the CUE domain did not change the turnover of PS1ΔCUE comparing to wild-type PS1 **(Figure 3.13)**, suggesting that separated from the endoproteolytic cleavage, level of full-length presenilin is tightly regulated by another system in which TRAF6 is probably involved. Collectively, regulation of TRAF6 on presenilin is independent of the CUE domain and turnover of the full-

length presenilin is independent on its endoproteolysis. Although it has been shown that CUE domain of presenilin is essential for its polyubiquitin binding, function of the CUE domain needs to be further explored.

The presenilin proteins undergo a variety of posttranslational modifications, which alter the functions of presenilins and their interaction with other proteins. For example, phosphorylation of the presenilins inhibits their cleavage by caspases and disrupts PS1 interaction with β-catenin (Walter et al., 1996a; Walter et al., 1999). Moreover, phosphorylation of PS1 is reported to affect turnover of PS1 and its NTF/CTF fragments as well as altering γ-secretase activity (Walter et al., 1996a; Walter et al., 1999). Modification such as polyubiquitination leads to the proteasomal degradation of presenilins (Kim et al., 1997b; Fraser et al., 1998; Marambaud et al., 1998). Ubiquilin involves in proteasome degradation pathway of presenilins by interacting with polyubiquitinated presenilin and preventing presenilin being targeted by proteasomal degradation (Mah et al., 2000; Massey et al., 2004). Moreover, it has been shown that mutation of two lysine residues in PS2 reduces its ubiquitination, results in the destabilization of PS2 and inhibits its binding to Ubiquilin, demonstrating the importance of ubiquitination modification for the stability and activity of PS2 (Ford and Monteiro, 2007). Only one of these two lysine residues is conserved in PS1 (K265). However, mutation of this single site did not cause any measurable change in ubiquitination or protein levels of PS1 (**Figure 3.23**).

TRAF6 has been shown to regulate various signalling events through the formation of Lys-63 linked polyubiquitin chains on different target proteins such as MALT1 (Sun et al., 2004; Oeckinghaus et al., 2007), NEMO (Sebban-Benin et al., 2007a; Rahighi et al.,

2009) or TRAF6 itself (Lamothe et al., 2007a). Similar to our observation that TRAF6 increases the level of presenilin and alters its turnover, another E3 ligase SEL-10 was reported to alter PS1 turnover by ubiquitinating PS1 (Lamothe et al., 2007a) which leads us to investigate PS1 as a substrate of TRAF6-mediated ubiquitination. We showed that PS1 is modified by TRAF6 through K63-linked polyubiquitination (**Figure 3.14**) and the ubiquitin-binding CUE domain of PS1 is not required for this modification. Furthermore we showed that PS1 is deficient in its ubiquitination in TRAF6 knock-out MEF cells and the full-length level of PS1 is decreased (**Figure 3.15**). Then we showed that PS1 ubiquitination is specifically regulated by TRAF family member that possess E3 ligase activity, but not by other irrelevant E3 ligases (**Figure 3.16**). Presenilin holoproteins and NTF/CTF fragments have been reported to have distinct subcellular distributions. Full-length presenilins are localized within the ER whereas the NTF and CTF are predominantly localized to the Golgi apparatus (Zhang et al., 1998). Therefore to identify which forms of presenilin are regulated by TRAF6 is important to understand the significance of this modification. We further showed that only full-length PS1 is the substrate of TRAF6-mediated ubiquitination, but not any of the fragments (**Figure 3.17**). Presenilin NTF/CTF heterodimer serves as the catalytic subunits of the γ-secretase complex. Consistent with the previous finding, TRAF6 was revealed not to interact with or affect any of the γ-secretase components, including Nicastrin, Pen-2 and Aph-1 (**Figure 3.19**). Additionally, we showed that over-expression of TRAF6 does not alter the γ-secretase cleavage of APP CT100, suggesting that γ-secretase activity is not regulated by TRAF6.

Full-length presenilins were reported to function as passive ER Ca^{2+} leak channels and maintain the calcium homeostasis of the ER, disruption of which has been linked

to the pathogenesis of AD (Tu et al., 2006). Familial AD-linked mutations have been shown to disrupt presenilin function as ER Ca^{2+} leak channels (Nelson et al., 2007). Additionally, transmembrane 7 and 9 of mouse PS1 were reported to be essential for forming the conductance pore of the calcium leak channels (Nelson et al., 2011). PS1 is reported to interact with sarco ER calcium-ATPase and expression of full-length PS1 is increased upon ER stress (Jin et al., 2010). However, little is known about how this full-length presenilin function is regulated in terms of the protein stability and the post-translational modification of full-length protein. As repression of PS1 transcription by JNK inhibitor suppresses the ER Ca^{2+} leak and considering the full-length levels of presenilins are regulated by TRAF6, we decided to examine if TRAF6 is a regulator of presenilin ER Ca^{2+} channels. Similar to presenilin knock-out MEF cells, we found that TRAF6 knock-out MEF cells are deficient in maintaining the size of the ER Ca^{2+} pool (**Figure 3.20**), suggesting that knock-out of TRAF6 attenuates functioning of the ER Ca^{2+} leak channels. Ca^{2+} signalling is an important factor in the osteoclast differentiation and TRAF6 is also reported to be essential for RANKL-mediated osteoclast differentiation (Lamothe et al., 2007b; Kajiya, 2012). Osteopetrosis observed in TRAF6 knock-out mice could potentially be linked to the deficiency in ER Ca^{2+} signalling (Lomaga et al., 1999b). We cannot yet prove that knock-out of TRAF6 disrupts ER homeostasis by affecting passive presenilin ER Ca^{2+} leak channels, however given that knock-out of TRAF6 decreases presenilin levels and TRAF6 stabilized full-length presenilin by inducing ubiquitination modification, we hypothesis that TRAF6 involves in the regulation of presenilin functioning as passive ER Ca^{2+} leak channels by stabilizing full-length presenilin through ubiquitination modification.

In conclusion, we report presenilins as novel substrates for TRAF6 induced lysine-63 linked polyubiquitination. Moreover, we demonstrate that TRAF6-mediated ubiquitination of presenilin manipulates presenilin stability, but not γ-secretase activity. TRAF6 may regulate presenilin function as passive ER Ca^{2+} leak channels, however further work is required to map the precise TRAF6-targeted sites of presenilin ubiquitination and to determine the functions and effects of presenilin ubiquitination.

Chapter 4:

RESULTS

TRAF6-induced polyubiquitination of IL-1R1 C-terminus is essential for its membrane expression and signalling transduction

Introduction:

Conventionally, TRAF6 is reported as an adaptor protein for the IL-1R1 signalling complex and is required for the association to IRAK1 and the recruitment of TAK1 complex by facilitating K63-linked polyubiquitination on the substrates or itself (Qian et al., 2001; Jiang et al., 2002; Ye et al., 2002). Our group previously reported IL-1R1 as a novel substrate of γ-secretase-dependent regulated intramembrane proteolysis and antagonism of γ-secretase activity was shown to impair responsiveness of IL-1R1 to IL-1β stimulation, indicating that γ-secretase cleavage of IL-1R1 may be a control mechanism for IL-1R1-mediated signalling (Elzinga et al., 2009b). Additionally, TRAF6 and IRAK2 have been shown to interact with PS1, revealing a potential role of TRAF6 in modulating the regulated intramembrane proteolysis of IL-1R1. In addition to regulated intramembrane proteolysis, IL-1R1 undergoes various post-translational modifications including glycosylation (Dower et al., 1989), ubiquitination (Brissoni et al., 2006) and phosphorylation (Gallis et al., 1989). Additionally, our group showed that IL-1R1 is a substrate for TRAF6-mediated ubiquitination and TRAF6 enhances regulated intramembrane proteolysis of IL-1R1 (Twomey et al., 2009). Ubiquitination of different receptors have been reported as important regulating events for receptor trafficking and signalling transduction. For instance, monoubiquitination of Notch receptor is required for its ligand-induced activation and γ-secretase cleavage (Gupta-Rossi et al., 2004b). TRAF6-mediated K63-linked polyubiquitination of the nerve growth factor receptor TrkA triggers its internalization and signalling (Geetha et al., 2005). IL-1R1 itself is ubiquitinated upon IL-1β stimulation and is subsequently coupled by Tollip to its lysosomal degradation (Brissoni et al., 2006). The biological relevance of γ-secretase cleavage of IL-1R1 and the role of the subsequently

generated IL-1R1 ICD are still unknown. However IL-1R1 nucleus translocation has been reported (Aveleira et al., 2010) which suggests the possibility that the γ-secretase generated IL-1R1 ICD may translocate to the nucleus. As mentioned above, TRAF6 is involved in the regulation of IL-1R1 regulated intramembrane proteolysis, studying and determining the regulatory mechanism controlling IL-1R1 cleavage may have significance in understanding the signal transduction potential of IL-1R1 ICD.

In this study we attempted to map the sites of TRAF6-mediated ubiquitination of IL-1R1 using different approaches including *in vitro* ubiquitination assay on IL-1R1 peptide arrays and site-directed mutagenesis of IL-1R1 lysine residues. Upon identifying IL-1R1 ubiquitination sites, we sought to study the functional importance of TRAF6-mediated ubiquitination of IL-1R1 in terms of IL-1R1 signalling transduction, cellular localisation and regulated intramembrane proteolysis.

4.1 TRAF6-induced polyubiquitination of IL-1R1 C-terminus is essential for its membrane localization and signalling transduction

4.1.1 Using Peptide array to map the sites of IL-1R1 ubiquitination

To further study the role of TRAF6 induced IL-1R1 ubiquitination, we began with mapping the sites of TRAF6-mediated ubiquitination of IL-1R1. Firstly, we attempted to reveal the potential ubiquitination sites by applying the combination of *in vitro* ubiquitination assay and peptide arrays. Briefly, the IL-1R1 peptide arrays are synthesized as overlapping 18-mer peptides covering the entire C-terminus sequence of IL-1R1 which were produced by automatic SPOT synthesis and synthesized on continuous Whatman cellulose membrane using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Rosbot ASS 222 (Intavis Bioanalytical Instruments) (**Figure 4.1**). The *in vitro* ubiquitination reagents including ATP, recombinant E1, E2, ubiquitin and GST-TRAF6K124R (or GST as negative control) were placed on the cellulose membrane of the IL-1R1 peptide array and were incubated at 37^{o}C for 1h. After the reaction, the peptide arrays were washed and subjected to anti-ubiquitin antibody to reveal the ubiquitination status of the IL-1R1 peptides. We detected specific signals for certain IL-1R1 peptides which could be considered as ubiquitination of certain lysine residues (**Figure 4.1**). However, negative control reaction for which TRAF6 was replaced by GST also gave similar signals. To verify the E3 ligase specificity of our recombinant TRAF6 protein, we carried out a series of optimization of the *in vitro* ubiquitination assay with PS1 and recombinant TRAF6 or immunoprecipitated TRAF6 as shown above **(Figure 3.20)**.

A1: IESRFYKHPFTCFAKNTH
A2: RFYKHPFTCFAKNTHGID
A3: KHPFTCFAKNTHGIDAAY
A4: FTCFAKNTHGIDAAYIQL
A5: ……

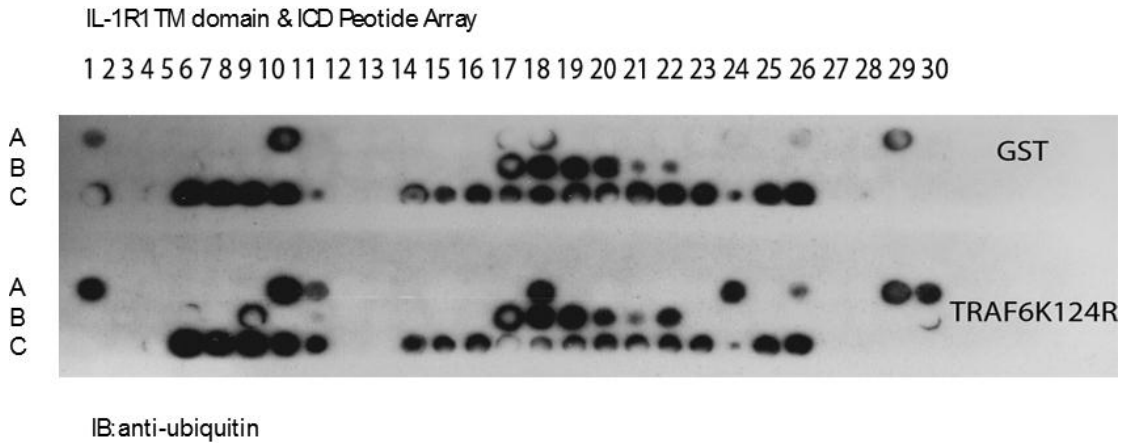

Figure 4.1 In vitro ubiquitination assay of IL-1R1 peptide arrays. Overlapping 18-mer peptides covering the entire C-terminus sequence of IL-1R1 were produced and synthesized on continuous cellulose membrane. The sequence of each peptide has 15 amino acids overlapped with the previous one and moves forward by 3 amino acids. IL-1R1 peptide arrays were incubated with 2μM E1, 0.5mg/ml E2, 50μM ubiquitin, 100unit/ml inorganic pyrophophatase, 50mM DTT, 0.1M Mg-ATP and 2μM GST-TRAF6 or GST in 50μl reaction solution at 37 ^{o}C for 1 hour. After reaction, IL-1R1 peptide arrays were washed in 0.2M NaOH stripping buffer to remove non-covalent binding. Ubiquitination status of the IL-1R1 peptide array was revealed by probing the membranes with anti-ubiquitin antibody.

4.1.2 Juxtamembrane domain of IL-1R1 contains potential ubiquitination sites

Have failed to map the IL-1R1 ubiquitination sites by *in vitro* approach, we decided to use mutagenesis approach and *in vivo* ubiquitination assay as an alternative plan. As there are 21 lysine residues in the intracellular domain of IL-1R1, we tried to narrow down the region containing potential ubiquitinated lysine residues by make IL-1R1 truncated mutants. Intracellular domain of IL-1R1 was divided into five regions each of which contains 3 to 5 lysine residues (**Figure 4.2**). Then we made five IL-1R1 truncated mutants by deleting one or multiple regions from the C-terminus (**Figure 4.2 lower graphic**). To test the ubiquitination status of these truncated mutants, HEK293T cells were transfected with wild-type IL-1R1 or these five truncations and HA-Ub and co-transfected with or without TRAF6. Thirty-six hours post-transfection, cells were harvested and cell lysates were subjected to immunoprecipitation for IL-1R1. Ubiquitination status of immunoprecipitated IL-1R1 was revealed by probing with anti-HA antibody. The shortest truncated mutant IL-1R1 (1-356) which lacks the entire intracellular domain showed dramatic reduction in the ubiquitination of IL-1R1 (**Figure 4.3**). However, the second shortest mutant IL-1R1 (1-390) showed a similar ubiquitination profile when compared to the IL-1R1, suggesting that the juxtamembrane domain (356-390) contains potential lysine residues for TRAF6-mediated ubiquitination. Analysis of the whole cell lysates by anti-IL-1R1 antibody revealed that all the full-length levels of the wild-type IL-1R1 and the truncations were increased by TRAF6 except for the shortest IL-1R1 (1-356), suggesting that the regulation of TRAF6 on IL-1R1 requires the juxtamembrane domain of IL1R1. Additionally, IL-1R1 (1-356) was detected as a single band in contrast to the multiple bands of wild-type or other truncations, indicating the importance of the

IL-1R1 Home Sapiens

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        10         20         30         40         50         60
MKVLLRLICF IALLISSLEA DKCKEREEKI ILVSSANEID VRPCPLNPNE HKGTITWYKD
        70         80         90        100        110        120
DSKTPVSTEQ ASRIHQHKEK LWFVPAKVED SGHYYCVVRN SSYCLRIKIS AKFVENEPNL
       130        140        150        160        170        180
CYNAQAIFKQ KLPVAGDGGL VCPYMEFFKN ENNELPKLQW YKDCKPLLLD NIHFSGVKDR
       190        200        210        220        230        240
LIVMNVAEKH RGNYTCHASY TYLGKQYPIT RVIEFITLEE NKPTRPVIVS PANETMEVDL
       250        260        270        280        290        300
GSQIQLICNV TGQLSDIAYW KWNGSVIDED DPVLGEDYYS VENPANKRRS TLITVLNISE
       310        320        330        340        350        360
IESRFYKHPF TCFAKNTHGI DAAYIQLIYP VTNFQKHMIG ICVTLTVIIV CSVFIYKIFK
       370        380        390        400        410        420
IDIVLWYRDS CYDFLPIKAS DGKTYDAYIL YPKTVGEGST SDCDIFVFKV LPEVLEKQCG
       430        440        450        460        470        480
YKLFIYGRDD YVGEDIVEVI NENVKKSRRL IIILVRETSG FSWLGGSSEE QIAMYNALVQ
       490        500        510        520        530        540
DGIKVVLLEL EKIQDYEKMP ESIKFIKQKH GAIRWSGDFT QGPQSAKTRF WKNVRYHMPV
       550        560
QRRSPSSKHQ LLSPATKEKL QREAHVPLG
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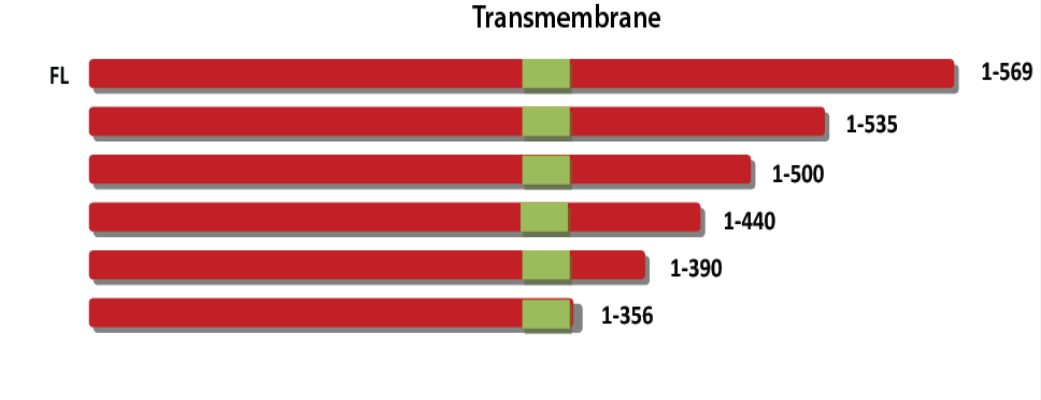

Figure 4.2 Amino acid sequence of human IL-1R1 and five IL-1R1 truncations. 21 lysine residues of the intracellular domain of IL-1R1 are labelled as blue letters. Transmembrane domain is marked with red letters. The intracellular domain of IL-1R1 is divided into five regions and these regions are highlighted by different colours. Lower schematic shows the wild-type IL-1R1 and the five truncated mutants which have one or several regions deleted from the C-terminus.

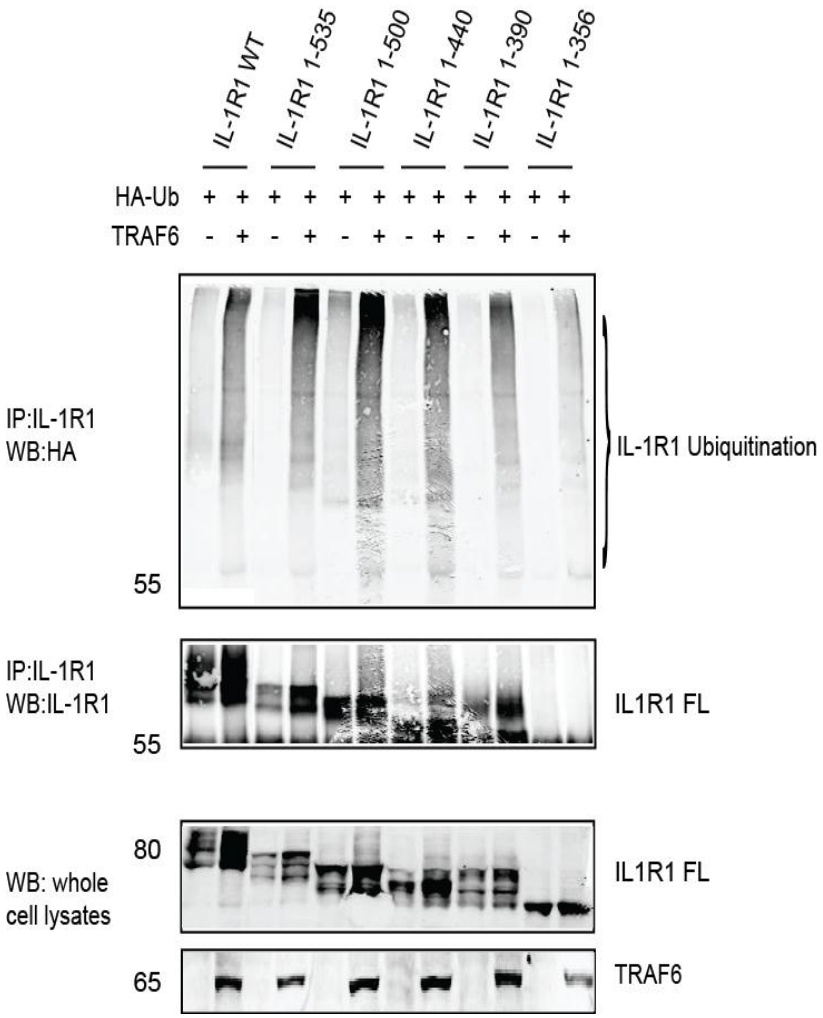

Figure 4.3 Juxtamembrane domain of IL-1R1 contains potential ubiquitination sites. HEK293T cells were transiently transfected with wild-type IL-1R1 or the other five IL-1R1 truncated mutants and HA-Ub and co-transfected with or without TRAF6 as indicated. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of IL-1R1. Precipitated IL-1R1 was detected by anti-IL-1R1 N-terminus antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated at least three times showing the similar results.

juxtamembrane domain for its post-translational modification, including perhaps its ubiquitination.

4.1.3 Identify IL-1R1 ubiquitination sites by mutagenesis of single or double lysine residues.

To map the sites for IL-1R1 ubiquitination, we mutated 15 lysine residues of IL-1R1 C-terminus domain respectively by making single or double lysine mutants, including the four lysine residues in the juxtamembrane domain and other potential lysine residues identified from the peptide array. Then we tested these mutants in the *in vivo* ubiquitination assay. HEK293T cells were transfected with wild-type IL-1R1 or IL-1R1 lysine mutants and co-transfected with HA-Ubiquitin alone or in combination with TRAF6. Thirty-six hours after transfection, cells were harvested under SDS denaturing condition and cell lysates were subjected to immunoprecipitation for IL-1R1. Ubiquitination status of immunoprecipitated IL-1R1 was revealed by probing with anti-HA antibody. Mutagenesis of any of the fifteen lysine residues alone did not cause any deficiency in TRAF6-induced ubiquitination of IL-1R1 (**Figure 4.4**), indicating the possibility that multiple lysine sites may be involved in IL-1R1 ubiquitination. Moreover, by over-expressing the wild-type IL-1R1 along with all the lysine mutants, we detected deficiencies in the IL-1R1 CTD level for some lysine mutants including IL-1R1 K378/383R, K445/446R, K527/532R and K548R (**Figure 4.5**), which supports our speculation that ubiquitination of IL-1R1 may occur on multiple lysine residues and ubiquitination of these lysine residues is important for the production or stability of IL-1R1 CTD.

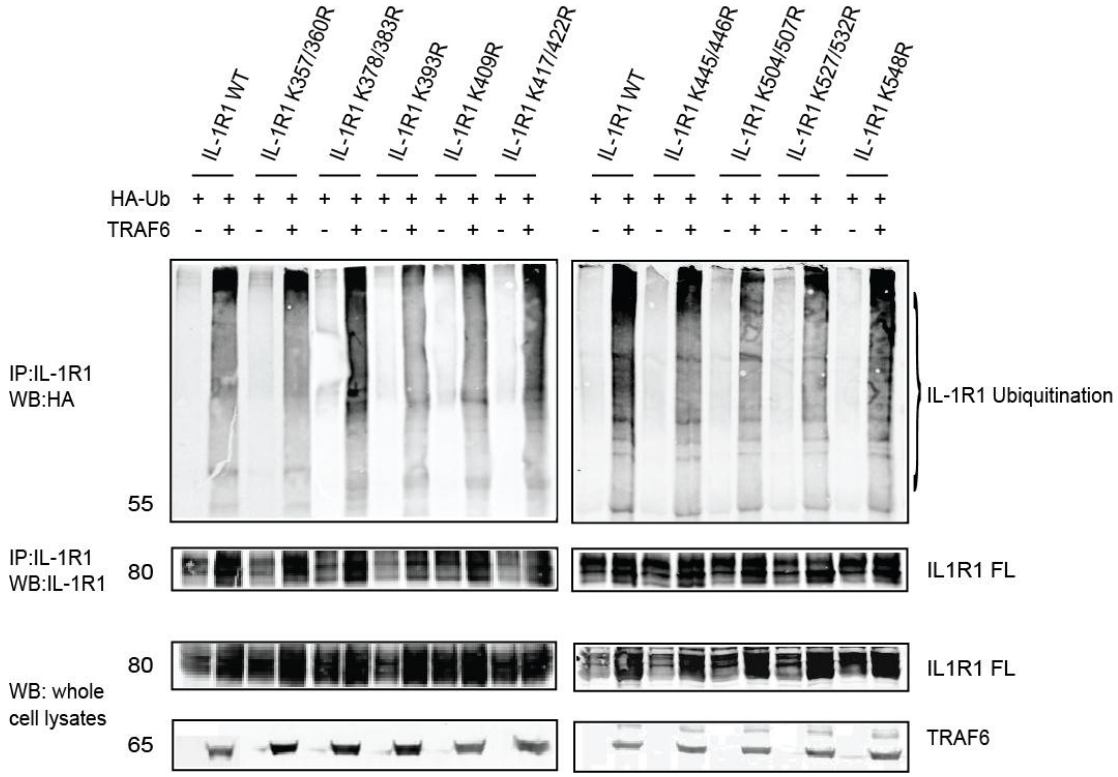

Figure 4.4 Single or double lysine mutation of IL-1R1 did not cause difference in IL-1R1 ubiquitination. HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 lysine mutants and co-transfected with HA-Ubiquitin or HA-Ubiquitin and TRAF6 as indicated. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. High molecular smears were detected with anti-HA epitope antibody revealing the ubiquitination status of IL-1R1. Precipitated IL-1R1 was detected by anti-IL-1R1 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated at least three times showing the similar results.

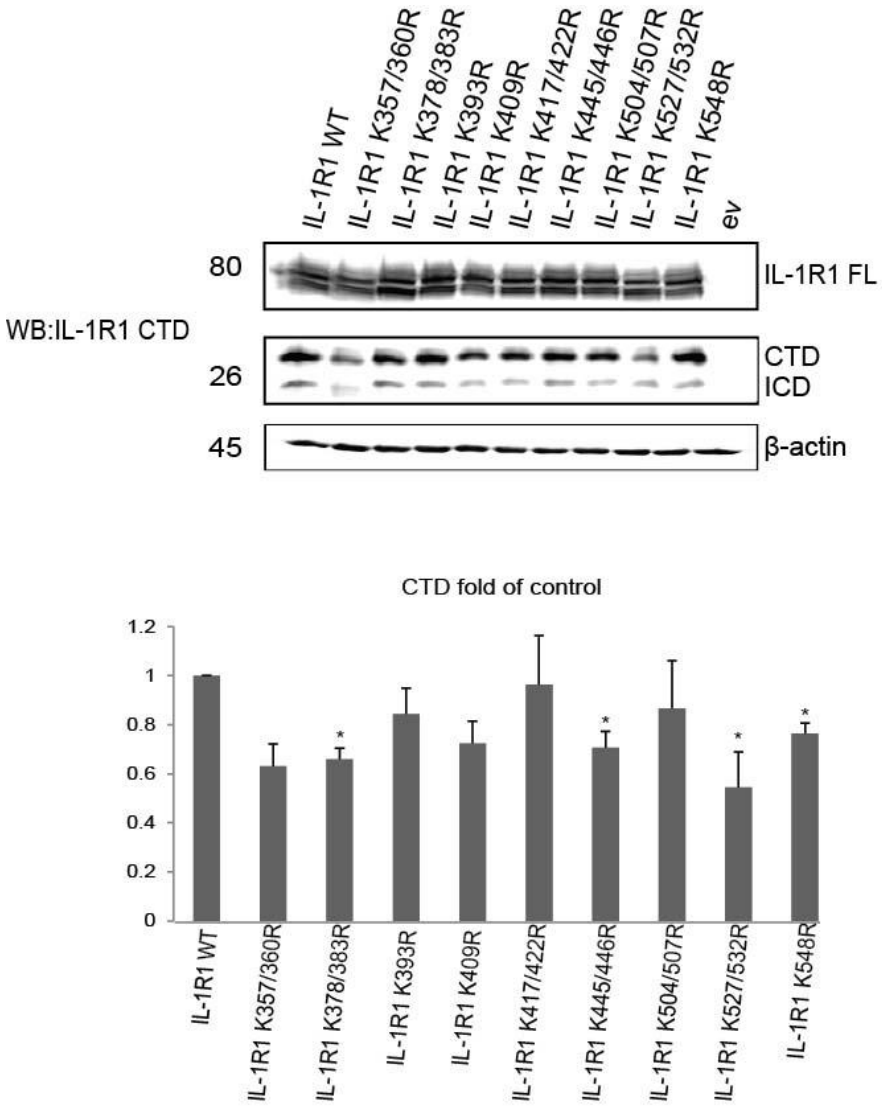

Figure 4.5 Mutagenesis of certain lysine residues caused deficiencies in the IL-1R1 CTD levels. HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 lysine mutants. Thirty-six hours post transfection cells were harvested and the cell lysates were subjected to Western blot with anti-IL-1R1 C-terminus antibody. Full-length IL-1R1 and IL-1R1 CTD and ICD were detected and measured by densitometry. CTD levels of wild-type IL-1R1 and IL-1R1 lysine mutants were normalised to their full-length protein levels respectively and then were shown as relative level of the wild-type IL-1R1 CTD. Experiment was repeated at least three times showing the similar results.

4.1.4 Mutagenesis of all four lysine residues in the juxtamembrane domain caused severe alteration to the IL-1R1 protein.

Having shown that the juxtamembrane domain of IL-1R1 may contain potential ubiquitination sites and ubiquitination of IL-1R1 may occur on multiple lysine residues, we mutated all four lysine residues in the juxtamembrane domain of IL-1R1. Then the IL-1R1 Quadra-lysine mutant was transfected in HEK293T cells as well as the wild-type IL-1R1 and the cell lysates were analysed by Western blot with anti-IL-1R1 C-terminus and N-terminus antibodies. Mutagenesis of all four lysine residues in the juxtamembrane domain appeared to cause severe alteration to the IL-1R1 protein and no protein was detected with an anti-IL-1R1 C-terminus antibody (**Figure 4.6**). When probing with an anti-IL-1R1 N-terminus antibody, no IL-1R1 full-length but only an truncated N-terminus fragment was detected suggesting that mutagenesis of all four lysine residues in the juxtamembrane domain causes severe change to IL-1R1 protein and this mutant cannot be used for further experiment.

4.1.5 Mutating IL-1R1 lysine 360/378/383 resulted in deficiency of CTD ubiquitination and reduction of the CTD level.

Because mutagenesis of all four lysine residues in the juxtamembrane domain caused severe alteration and resulted in a non-functional mutant, we decided to take one step back and mutate only three lysine residues in the juxtamembrane domain. So we made three IL-1R1 lysine mutants with the first three (K356/360/378), last three (K360/378/383) or the middle two (K360/378) lysine residues of the juxtamembrane domain mutated. These IL-1R1 lysine mutants were then transfected

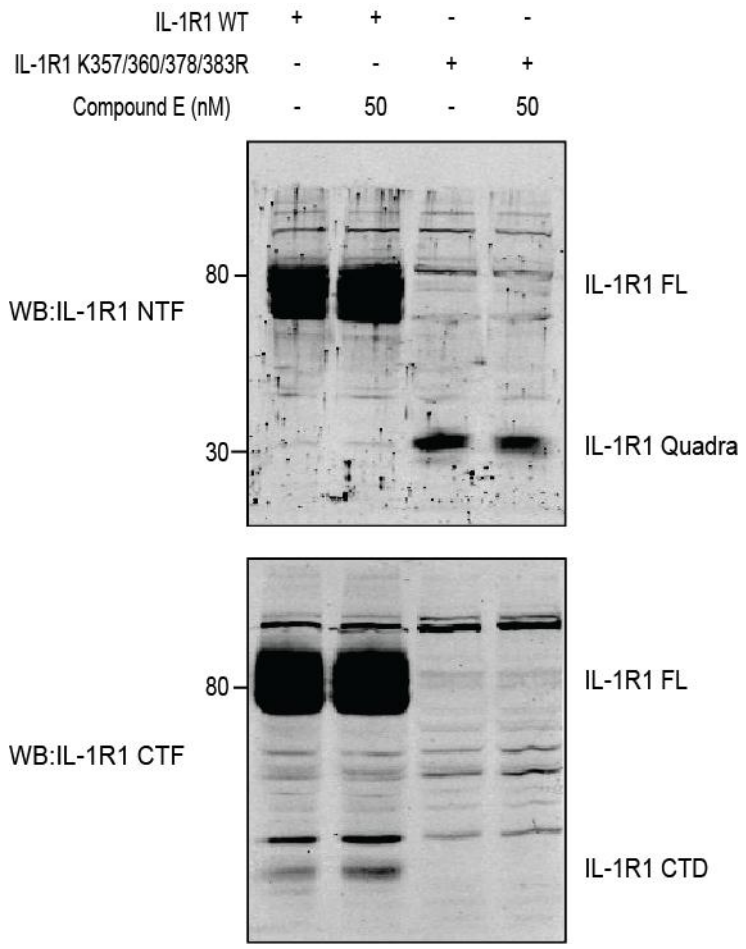

Figure 4.6 Mutagenesis of all four lysine residues in the juxtamembrane domain caused severe alteration to the IL-1R1 protein and resulted in a non-functional mutant.HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 K357/360/378/383R mutant. Twenty-four hours post transfection selected samples were treated with 50nM Compound E for 12 hours. Then all cells were harvested and the cell lysates were subjected to Western blot with anti-IL-1R1 N-terminus and anti-IL-1R1 C-terminus antibodies. Experiment was repeated two times showing the similar results.

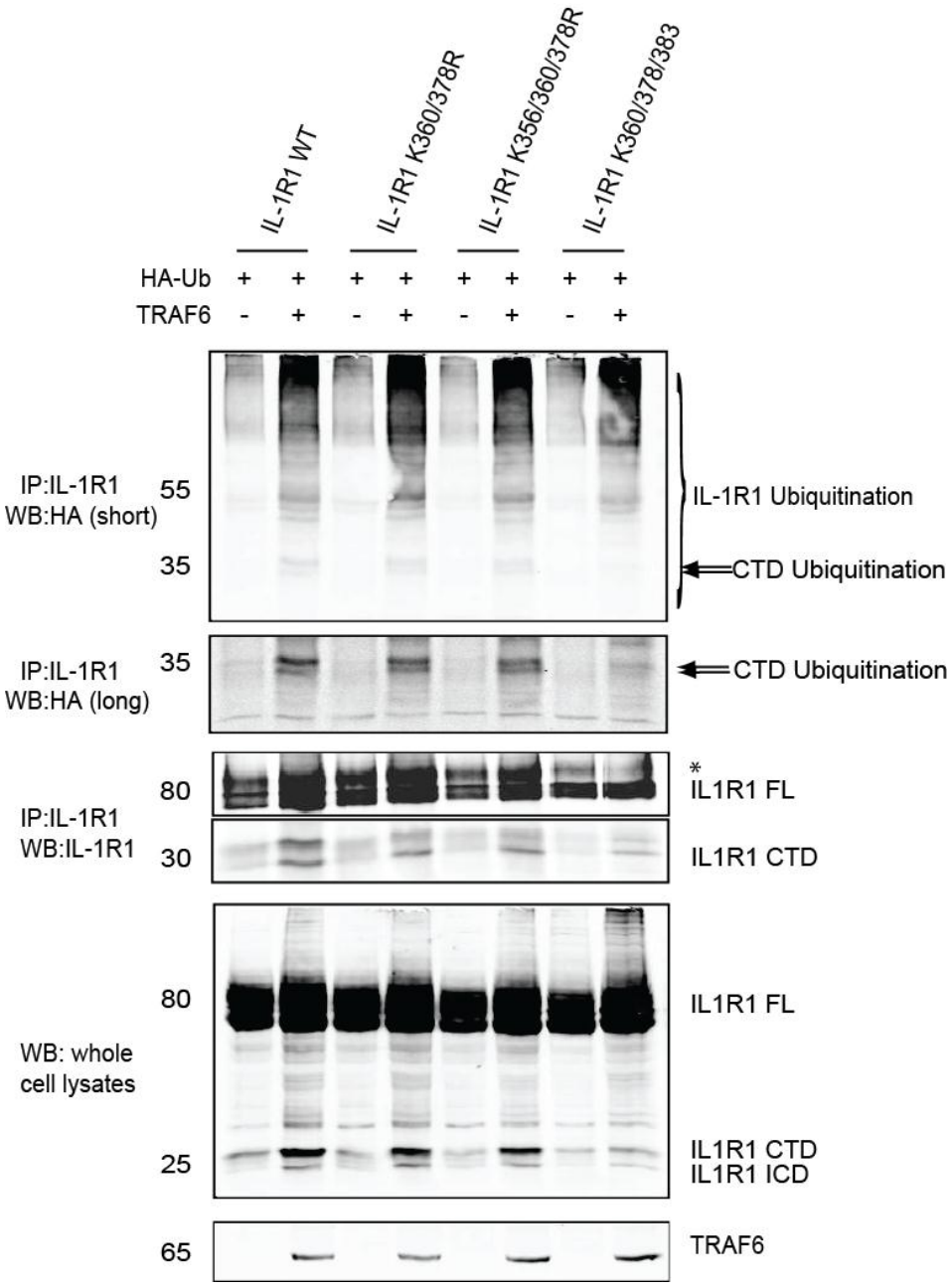

Figure 4.7 Mutating IL-1R1 lysine 360/378/383 resulted in deficiency of CTD ubiquitination and reduction of the CTD level. HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 double/triple lysine mutants and co-transfected with HA-Ubiquitin or HA-Ubiquitin and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. Ubiquitinated IL-1R1 full-length or CTD were detected with anti-HA antibody. Precipitated IL-1R1 was detected by anti-IL-1R1 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated at least three times showing the similar results.

in HEK293T cells and co-transfected with HA-ubiquitin or HA-ubiquitin and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. TRAF6 induced poly-ubiquitination of the wild-type IL-1R1 as well as the lysine mutants which were detected as similar high-molecular-weight smears (**Figure 4.7**). However, TRAF6-induced low-molecular-weight double bands (**Figure 4.7 arrows**) which is considered to be ubiquitinated IL-1R1 CTD, was not detected in cell lyasates expressing the IL-1R1 K360/378/383R mutant, suggesting these three lysine residues could be the ubiquitination sites for IL-1R1 CTD ubiquitination. Furthermore, when the whole cell lysates were analysed by Western blot for IL-1R1, IL-1R1 K360/378/383R mutant showed an obvious reduction in detectable levels of IL-1R1 CTD when compared to CTD levels of all other IL-1R1 constructs (**Figure 4.7 lower panel**). However, full-length level of IL-1R1 K360/378/383R mutant was similar to the full-length level of other IL-1R1 constructs, which is still increased by TRAF6. Notably, immunoprecipitated wild-type full-length IL-1R1 was detected as double bands both of which were enhanced by TRAF6, whereas the upper band of IL-1R1 K360/378/383R was not altered by TRAF6 (**Figure 4.7 asterisk)**, indicating the deficiency of the mutant in TRAF6-induced modification**.** Interestingly, similar deficiency has also been detected with IL-1R1 K527/532R mutant (**Figure 4.4 second panel**).

4.1.6 Mutating IL-1R1 lysine 360/378/383/527/532 resulted in further reduction in the CTD level and deficiency of CTD ubiquitination.

As mentioned above, the observed reduction in generation of the IL-1R1 CTD and reduced detection of the highest molecular form of the full-length IL-1R1 when

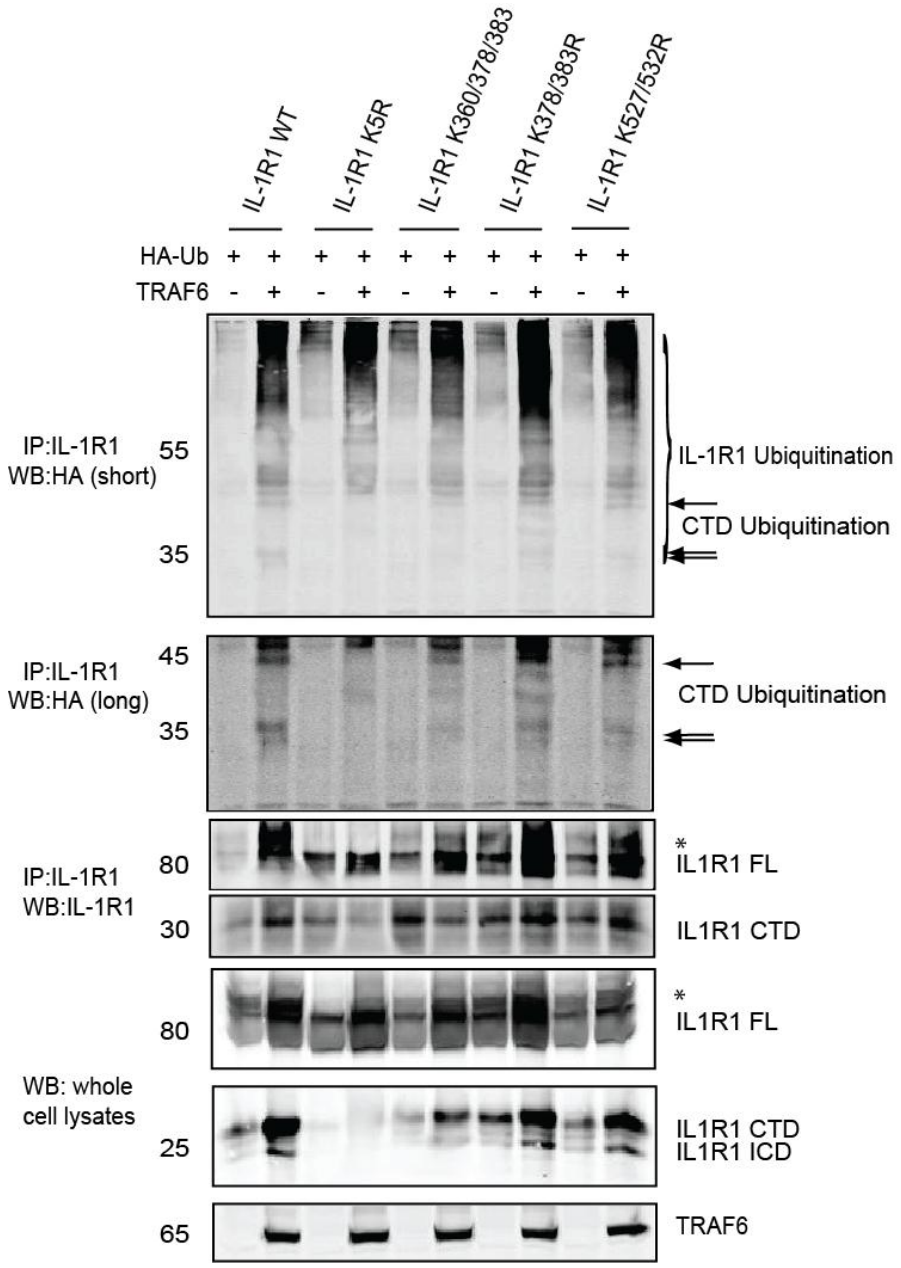

Figure 4.8 Mutating IL-1R1 lysine 360/378/383/527/532 resulted in further reduction in the CTD level and deficiency of CTD ubiquitination. HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 double/triple/penta lysine mutants and co-transfected with HA-Ubiquitin or HA-Ubiquitin and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. Ubiquitinated IL-1R1 full-length or CTD were detected with anti-HA antibody. Precipitated IL-1R1 was detected by anti-IL-1R1 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated at least two times showing the similar results.

lysine 360/378/383 residues were mutated have also been seen with another double lysine mutant, IL-1R1 K527/532R. Although these two groups of lysine residues are quite separated in the protein sequence, giving that the three-dimensional structure of IL-1R1 C-terminus is still unknown, these two domains could have close spatial localization and involve in the same TRAF6-mediated ubiquitination. So we further mutated lysine residues K527 and K532 and tested the IL-1R1 K360/378/383/527/532R (IL-1R1 K5R) mutant in the *in vivo* ubiquitination assay. The IL-1R1 K5R mutant was transfected along with other IL-1R1 double/triple lysine mutants in HEK293T cells and co-transfected with HA-ubiquitin alone or HA-ubiquitin and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. High-molecular-weight ubiquitination was still similar for the wild-type IL-1R1 and the lysine mutants. However, in addition to the diminished low molecular weight ubiquitinated double bands observed with IL-1R1 K360/378/383R mutant, another ubiquitinated IL-1R1 CTD band was abolished in cells expressing the IL-1R1 K5R mutant (**Figure 4.8 arrows**). Furthermore, the IL-1R1 CTD and ICD of the IL-1R1 K5R mutant could hardly be observed even with over-expression of TRAF6 (**Figure 4.8 lower panel**), suggesting TRAF6-targeted C-terminus lysine residues are further mutated. Notably, the highest molecular weight form of the full-length IL-1R1 was dramatically diminished in cells expressing the IL-1R1 K5R mutant (**Figure 4.8 asterisk**), suggesting its deficiency in TRAF6-induced regulation which could have significant effect on the functions of the receptor.

4.1.7 IL-1R1 CTD is poly-ubiquitinated by TRAF6.

To verify that the IL-1R1 CTD is ubiquitinated by TRAF6, we performed an *in vivo* ubiquitination assay on an IL1-R1 extracellular domain truncated mutant (IL-1R1 CTF) which contains a small part of the extracellular domain, the transmembrane domain and intracellular domain. Also to find out the type of ubiquitination occuring, we replace wild-type ubiquitin with a ubiquitin lysine-all-mutated mutant (HA-UbK0) which has all seven lysine residues mutated to arginine and can only form monoubiquitination but not polyubiquitination of the substrates. HEK293T cells were transfected with IL-1R1 or the IL-1R1 K5R mutant and co-transfected with HA-ubiquitin or HA-UbK0 and co-transfected with TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. With the over-expression of HA-UbK0, TRAF6 did not induce ubiquitination of IL-1R1 (**Figure 4.9**), suggesting that TRAF6 only mediates polyubiquitination of IL-1R1. Reprobing the blot with an anti-polyubiquitination antibody confirmed that TRAF6 induced polyubiquitination of IL-1R1 only in the presence of wild-type ubiquitin. Immunoprecipitated IL-1R1 CTF showed increased smear and ladders with over-expression of TRAF6 (**Figure 4.9 hash**), suggesting that the C-terminus region of IL-1R1 is ubiquitinated by TRAF6. Interestingly, co-expression of IL-1R1 and UbK0 mutant increased the levels of IL-1R1 CTD and especially the ICD, indicating that polyubiquitination of these fragments may be required for their degradation.

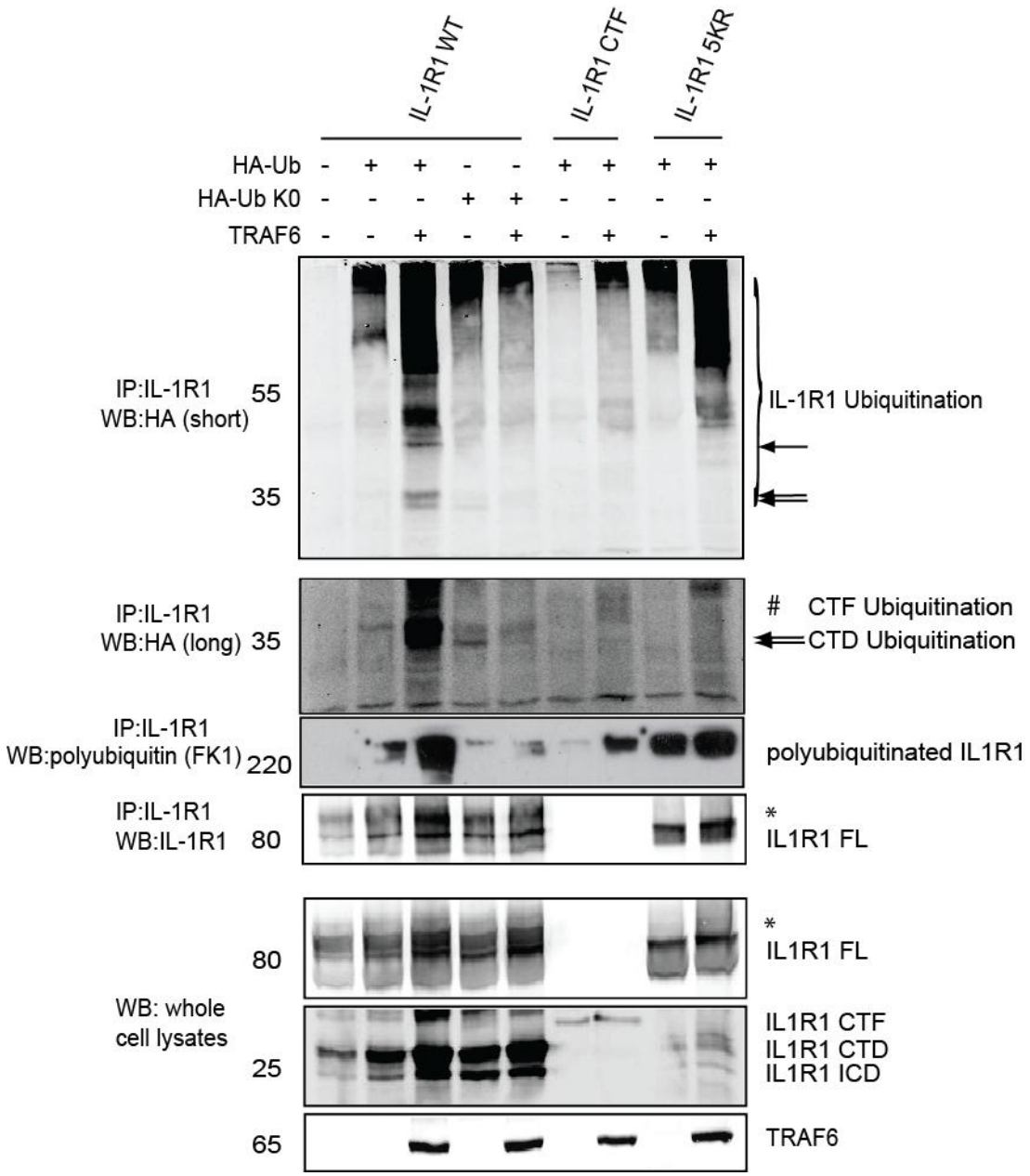

Figure 4.9 IL-1R1 CTD is poly-ubiquitinated by TRAF6. HEK293T cells were transiently transfected with wild-type IL-1R1 or IL-1R1 K5R and co-transfected with HA-ubiquitin or HA-UbK0 and co-transfected with TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for IL-1R1 and Western blot for HA-Ub. Ubiquitinated IL-1R1 full-length or CTD were detected with anti-HA antibody. Precipitated IL-1R1 was detected by anti-IL-1R1 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins. Experiment was repeated at least two times showing the similar results.

4.1.8 Mutagenesis of IL-1R1 C-terminus lysine residues leads to deficiency of IL-1R1 cell surface localization.

Having shown that mutagenesis of IL-1R1 C-terminus residues results in a reduction in CTD level and deficiency in C-terminus ubiquitination, next we tried to investigate the effect of mutagenesis on the membrane localization of IL-1R1. HEK293T cells were transfected with empty vector, wild-type IL-1R1, IL-1R1 K5R or IL-1R1 K360/378/383R (K3R) mutants. Forty-eight hours after transfection, cells were detached under non-permeabilization conditions and incubated with primary anti-IL-1R1 antibody for 45 minutes and secondary green fluorescent antibody for 30 minutes. After washings, levels of membrane IL-1R1 were measured by flow cytometry. HEK293T cells over-expressing IL-1R1 showed that more than 50% of the cells exhibited surface localization of IL-1R1, which was reduced by half when IL-1R1 K5R was over-expressed (**Figure 4.10**). Over-expression of IL-1R1 K3R only caused subtle reduction in cell surface levels of IL-1R1 when compared to that of wild-type IL-1R1 expressing cells. Collectively, this data suggests that mutagenesis of TRAF6-targeted IL-1R1 C-terminus lysine residues results in decreased IL-1R1 cell surface localization.

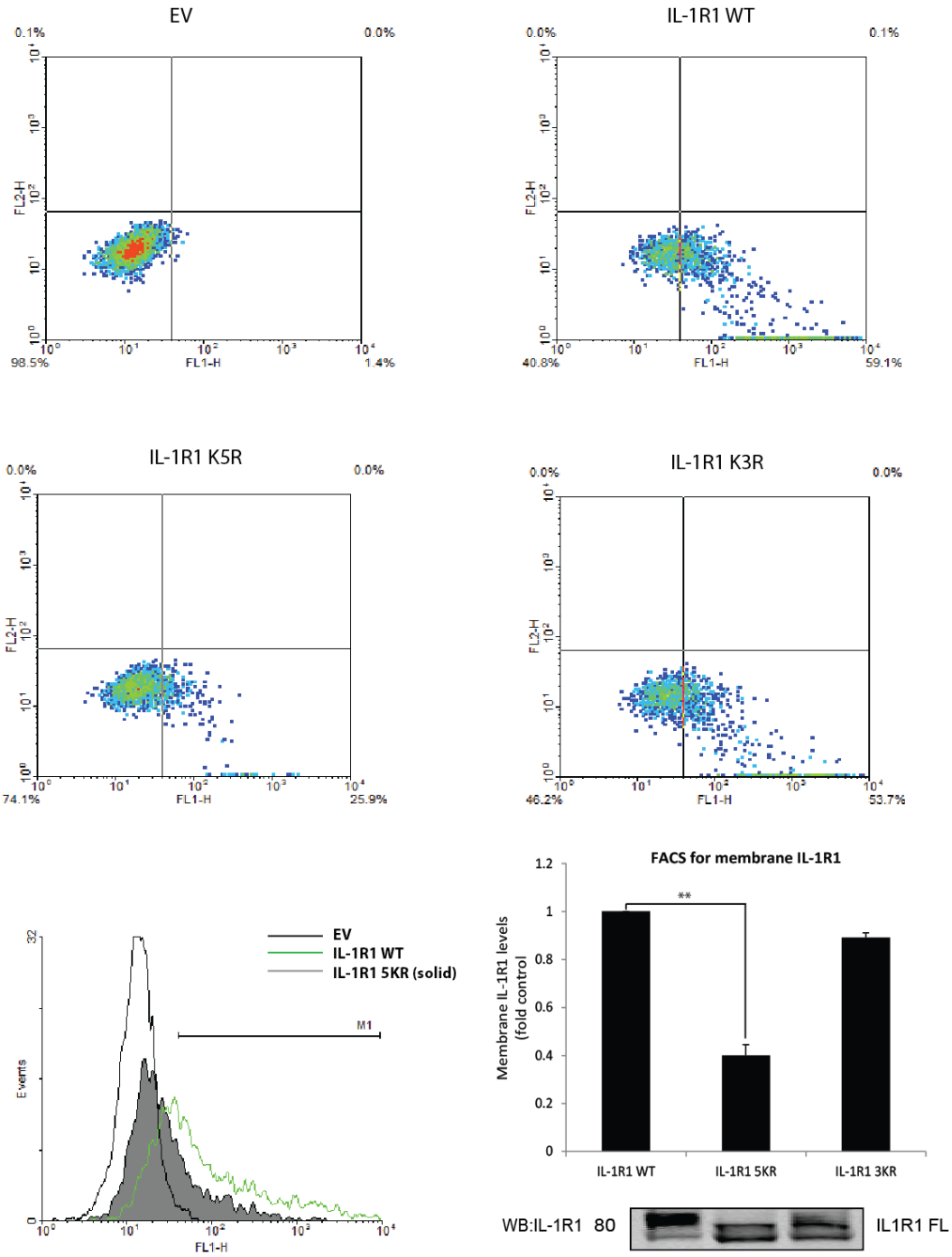

Figure 4.10 Mutagenesis of IL-1R1 C-terminus lysine residues leads to deficiency of IL-1R1 cell surface localization. HEK293T cells were transfected with empty vector, wild-type IL-1R1, IL-1R1 K5R, IL-1R1 K360/378/383R (K3R) mutants. 48 hours after transfection, cells were detached under nonpermeabilized condition and incubated with primary anti-IL-1R1 antibody for 45 minutes and secondary green fluorescent antibody for 30 minutes. After washings, levels of membrane IL-1R1 were measured by flow cytometry and shown as density plot and histogram graphs. Bars represent mean levels of surface IL-1R1 +SEM (n=3). Western blot of the cell lysates confirmed similar expression of all transfected constructs.

4.1.9 Mutagenesis of IL-1R1 C-terminus lysine residues causes attenuated NF-κB activation.

Having shown that IL-1R1 is ubiquitinated by TRAF6, we next examined the IL-1R1 downstream signalling events, namely NF-κB activation and the effects that mutagenesis of potential ubiquitination sites may have on subsequent signalling events. Firstly we optimized the NF-κB luciferase gene reporter assays to avoid any artificial events due to overexpression of IL-1R1. HEK293T cells were transfected with a NF-κB-dependent luciferase reporter construct and co-transfected with empty vector or increasing amount of wild-type IL-1R1 or IL-1R1 K5R. Forty-eight hours after transfection, cells were harvested and luciferase activities of the cell lysates were measured. Over-expression of IL-1R1 induced a dose-dependent increase in NF-κB activation. In contrast, lysates from cells expressing IL-1R1 K5R mutant had reduced NF-κB activation (**Figure 4.11 A**). Notably, the amount of over-expressed wild-type IL-1R1 was linearly corresponding to the activation level of NF-κB whereas over-expression of IL-1R1 K5R was saturated after more than 0.6μg DNA was transfected. Therefore, we decided to transfect 0.6μg DNA per well for the future experiments.

To further examine IL-1R1 and IL-1R1 K5R mediated NF-κB activation, we tested IL-1β-induced NF-κB activation in cells expressing wild-type IL-1R1 or IL-1R1 K5R expression constructs. HEK293T cells were transfected with a NF-κB-dependent luciferase reporter construct and co-transfected with empty vector, wild-type IL-1R1 or IL-1R1 K5R expression vector. Twenty-four hours after transfection, cells were serum starved overnight in serum-free medium. Then one set of the cells were

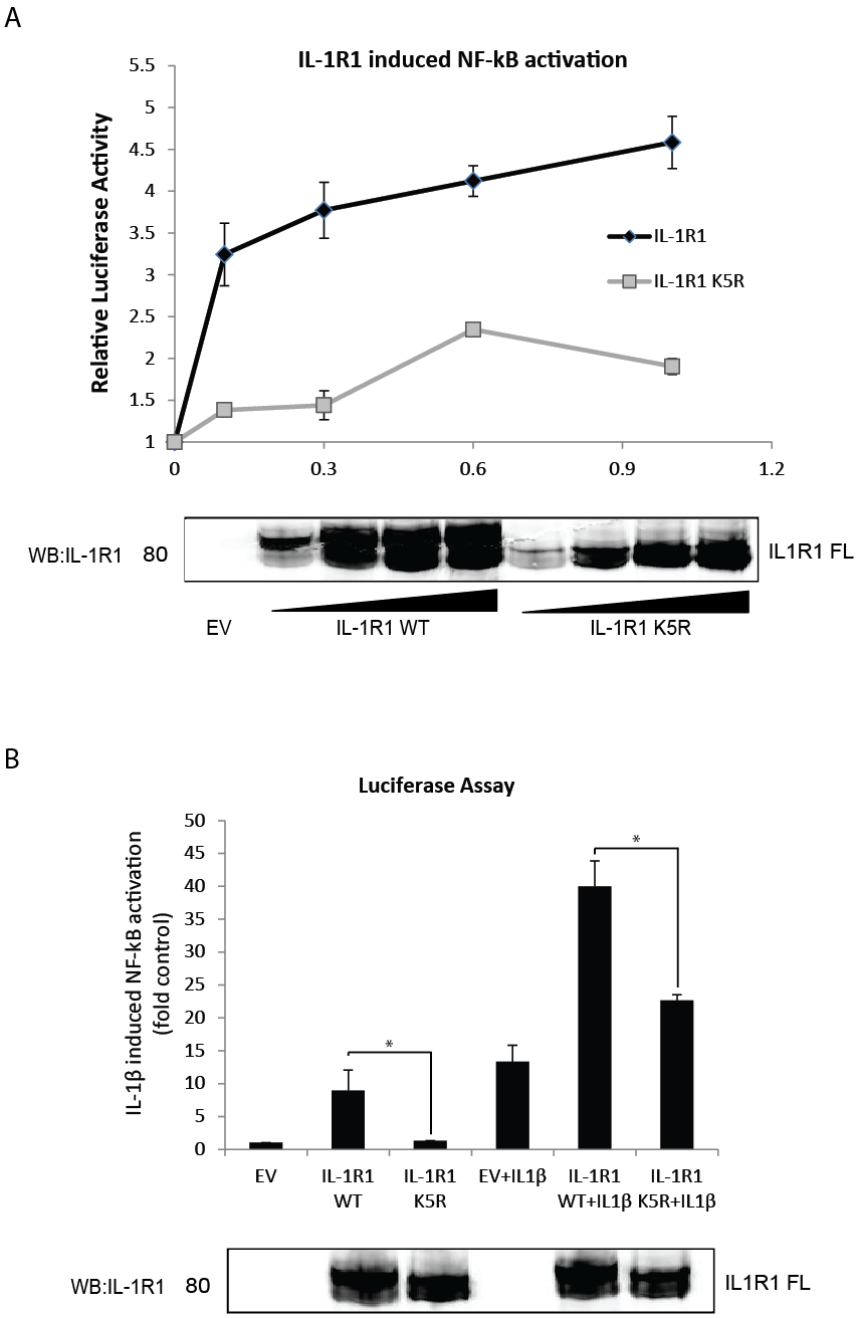

Figure 4.11 Mutagenesis of IL-1R1 C-terminus lysine residues causes attenuated NF-κB activation. A. HEK293T cells were transfected with a NF-κB-dependent luciferase reporter construct and co-transfected with empty vector or increasing amount of wild-type IL-1R1 or IL-1R1 K5R. Forty-eight hours after transfection, cells were harvested and luciferase activity of the cell lysates were measured by luminometer. Cell lysates were also analysed by Western blot to confirm the expression of all transfected constructs. **B.** HEK293T cells were transfected with a NF-κB-dependent luciferase reporter construct and co-transfected with empty vector, wild-type IL-1R1 or IL-1R1 K5R as duplication. 24 hours after transfection, cells were serum starved overnight and then one set of the cells were treated with 10nM IL-1β for six hours. Cells were then harvested and luciferase activity was measured.

treated with IL-1β for six hours and the other set were left untreated. After treatment, cells were harvested and luciferase activities were measured. Consistent with **Figure 4.11A**, over-expression of IL-1R1 induced activation of NF-κB while expression of IL-1R1 K5R mutant induced lower NF-κB activation (**Figure 4.11 B**). IL-1β stimulation lead to a robust activation of NF-κB in all cell cultures, however, cells expressing IL-1R1 K5R mutant showed a dramatic reduction in the NF-κB activation when compared to cells expressing wild-type IL-1R1. However, IL-1R1 K5R induced higher NF-κB activation than the empty vector, suggesting that this mutant is still functional but to a lesser extent. Collectively, mutagenesis of TRAF6-regulated IL-1R1 lysine residues causes attenuated NF-κB activation.

4.1.10 Mutagenesis of IL-1R1 C-terminus lysine residues results in deficiency in IL-1R1 CTD production rather than rapid CTD degradation.

IL-1R1 full-length and CTD have been shown to undergo lysosomal degradation after its internalisation (Brissoni et al., 2006). IL-1R1 is targeted through its ubiquitination modification by a CUE domain containing protein Tollip for lysosomal degradation (Brissoni et al., 2006). So any change in IL-1R1 ubiquitination could cause alteration in its degradation. To interpret the decreased CTD level caused by mutating of the IL-1R1 C-terminus lysine residues, we proposed two putative mechanisms for TRAF6 mediated IL-1R1 regulation: 1) TRAF6-regulated lysine residues of IL-1R1 are critical for its membrane localization and its internalisation after which the extracellular domain shedding and γ-secretase cleavage occur (results shown by other group member). Thus deficiency in IL-1R1 membrane localization and internalization precludes IL-1R1 ectodomain shedding and production of IL-1R1 CTD. 2) TRAF6

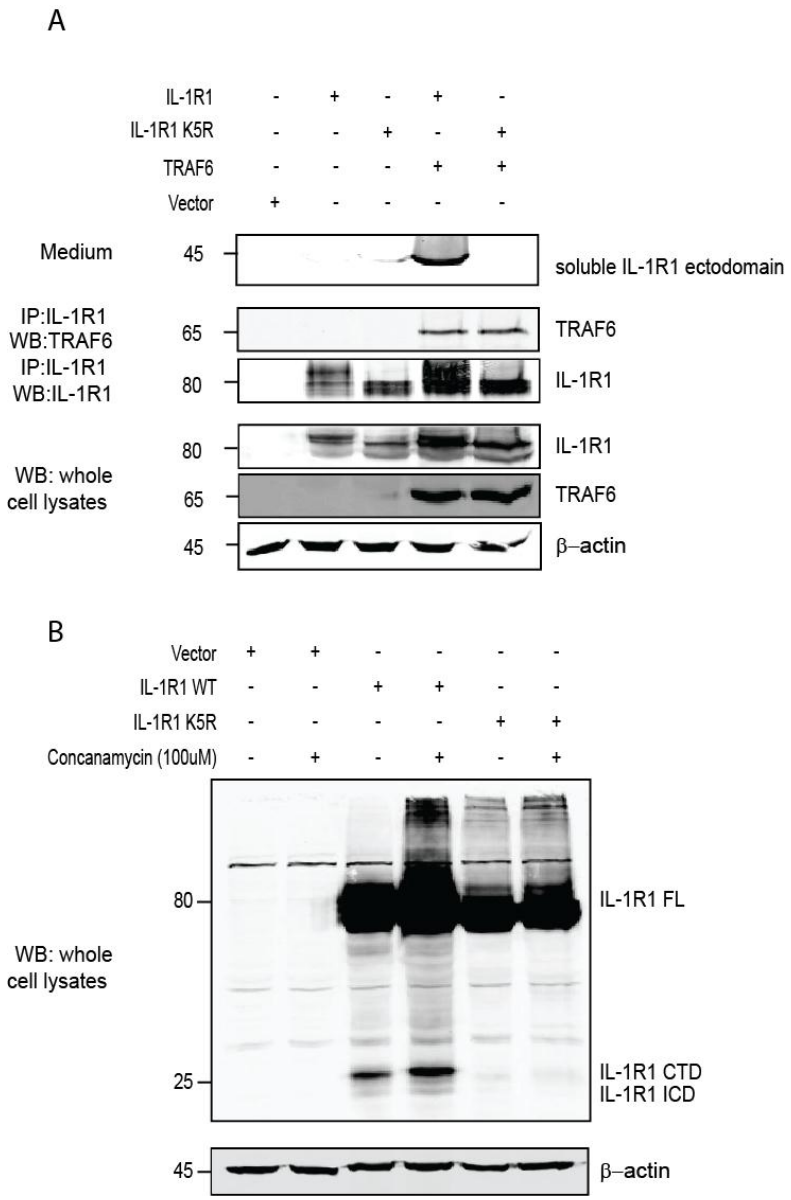

Figure 4.12 Mutagenesis of IL-1R1 C-terminus lysine residues results in deficiency in IL-1R1 CTD production rather than rapid CTD degradation. (**A**) HEK293T cells were transfected with IL-1R1 or IL-1R1 K5R mutant alone or co-transfected with TRAF6. Thirty-six hours after transfection, cell culture medium was collected and was subjected to Western blot with an anti-IL-1R1 N-terminus antibody. Cells were harvested under non-denaturing conditions and cell lysates were subjected to immunoprecipitation for IL-1R1 and then Western blot for TRAF6. Whole cell lysates were also analyzed by Western blot to confirm expression of all transfected plasmids (**B**) HEK293T cells were transfected with empty vector, wild-type IL-1R1 or IL-1R1 K5R as duplication. Twenty-four hours after transfection, one set of cells were treated with 100uM concanamycin for twelve hours in serum-free medium. Then cells were harvested and analysed for IL-1R1 by Western blot. B-actin levels were also measured to confirm equal loading. Experiment was repeated three time showing similar results.

stabilizes IL-1R1 CTD by inducing a different type of ubiquitination which is not targeted by lysosomal degradation. When the TRAF6 regulated sites within IL-1R1 CTD are mutated, IL-1R1 CTD could only be modified and targeted by the degradative pathway which caused rapid degradation of IL-1R1 CTD.

To test our hypothesis, firstly we investigated the TRAF6-induced ectodomain shedding of IL-1R1 and IL-1R1 K5R mutant. HEK293T cells were transfected with IL-1R1 or IL-1R1 K5R mutant alone or co-transfected with TRAF6. Thirty-six hours after transfection, cell culture medium was collected and was subjected to Western blot probing by an anti-IL-1R1 N-terminus antibody. Over-expression of TRAF6 induced dramatic increase in the detection of soluble IL-1R1 ectodomain (**Figure 4.12 A**). In contrast, TRAF6 did not induce any detectable ectodomain shedding in cells expressing IL-1R1 K5R mutant, suggesting that mutagenesis of IL-1R1 C-terminus lysine residues results in deficiency in IL-1R1 ectodomain shedding, thus prevents the IL-1R1 CTD production. Cells were also harvested under non-denaturing conditions and cell lysates were subjected to immunoprecipitation for IL-1R1 and Western blot for TRAF6. Co-immunoprecipitation analysis revealed that interaction between IL-1R1 and TRAF6 is not affected by mutagenesis of IL-1R1 C-terminus lysine residues.

To test the second hypothesis, we applied a lysosomal inhibitor concanamycin to check if inhibition of lysosomal degradation could rescue the CTD level of IL-1R1 K5R mutant. HEK293T cells were transfected with empty vector, wild-type IL-1R1 or IL-1R1 K5R as duplication. Twenty-four hours after transfection, one set of cells were treated with concanamycin (100uM) for twelve hours in serum-free medium. Cells were then harvested and analysed for IL-1R1 by Western blot. Full-length and CTD

levels of wild-type IL-1R1 were increased with concanamycin treatment, confirming that the full-length IL-1R1 and CTD are targeted by lysosomal degradation (**Figure 4.12**). However, the full-length level of IL-1R1 K5R mutant was only slightly increased and the CTD level remained the same with concanamycin treatment, suggesting that mutagenesis of IL-1R1 lysine residues does not cause rapid CTD degradation. Collectively, mutagenesis of IL-1R1 C-terminus lysine residues results in deficiency of IL-1R1 ectodomain shedding and precludes the production of IL-1R1 CTD.

4.1.11 TRAF6-mediated TLR4 ubiquitination and TRAF2-mediated TNFR1 ubiquitination.

TRAF6 is also essential for TLR4 signalling transduction and TLR4 signalling complex shares similar scheme as that of IL-1R1 (Verstak et al., 2009). However TNFR1 signalling complex recruits another TRAF protein, TRAF2, that is reported to have an inhibitory effect on TNFR1 signals (Nguyen et al., 1999). To expand our understanding about TRAF family protein mediated regulation of other receptors, we investigated if TLR4 and TNFR1 are regulated by TRAF6 and TRAF2 respectively. Firstly, HEK293T cells were transfected with TLR4 and co-transfected with HA-Ub and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for TLR4 and Western blot for HA-Ub. Ubiquitination of TLR4 was clearly detected as a high-molecular-weight smear (**Figure 4.13**). Additionally, over-expression of TRAF6 appeared to increase the levels of TLR4 full-length and CTD. Our result suggests that TLR4 may undergo TRAF6-mediated regulation, similar to IL-1R1.

Secondly, TRAF2 and TRAF6 mediated regulation of TNFR1 was also tested. HEK293T cells were transfected with TNFR1 and co-transfected with HA-Ub and TRAF2,

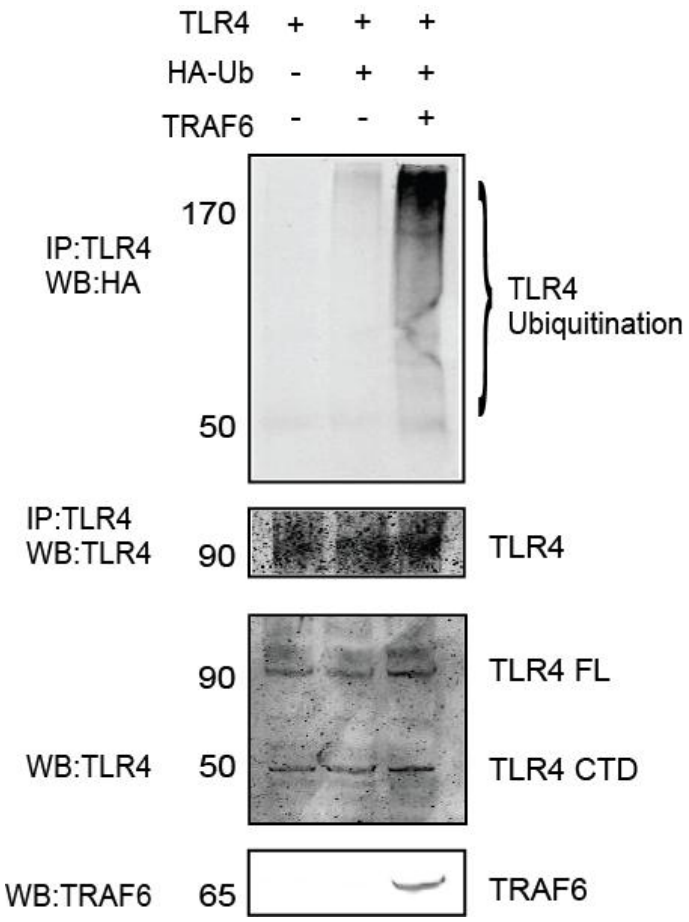

Figure 4.13 TRAF6-mediated ubiquitination of TLR4. HEK293T cells were transiently transfected with with TLR4 and co-transfected with HA-Ub and TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for TLR4 and Western blot for HA-Ub. Ubiquitinated TLR4 was detected with anti-HA antibody. Precipitated TLR4 was detected by anti-TLR4 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins.

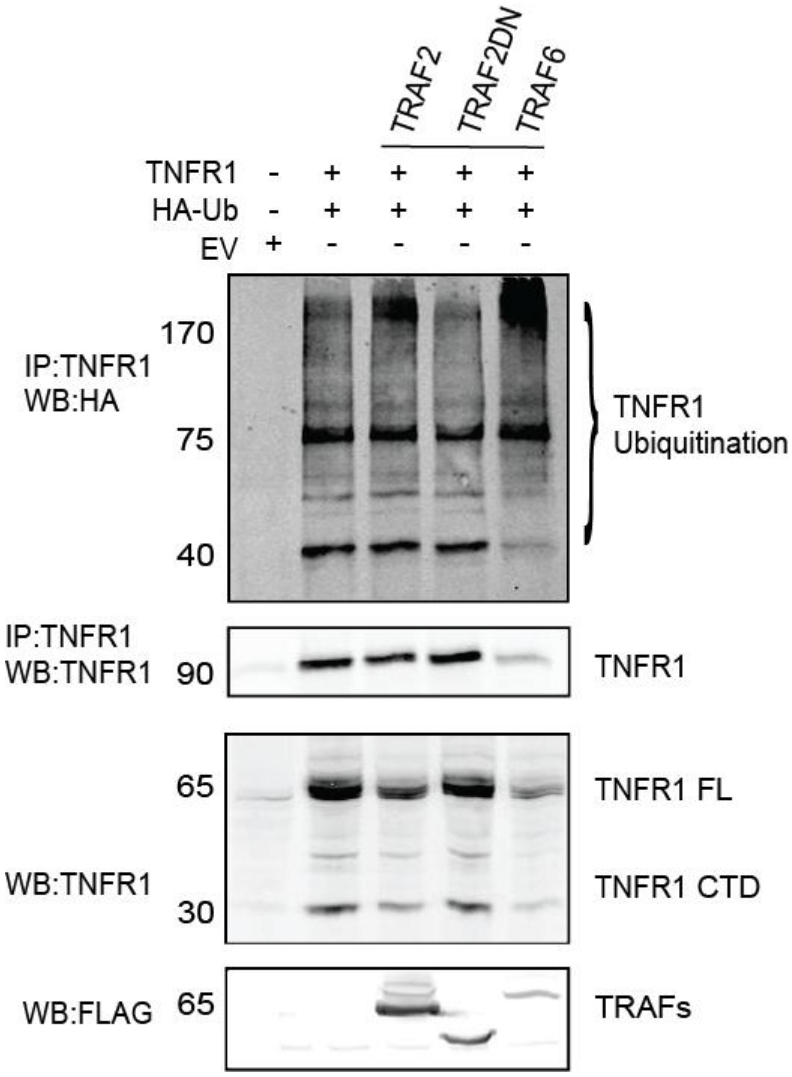

Figure 4.14 TRAF2 and TRAF6 mediated ubiquitination of TNFR1. HEK293T cells were transiently transfected with TNFR1 and co-transfected with HA-Ub and TRAF2, TRAF2DN or TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for TNFR1 and Western blot for HA-Ub. Ubiquitination of TNFR1 was detected with anti-HA antibody. Precipitated TNFR1 was detected by anti-TNFR1 antibody. Western blot of whole cell lysates confirms expressions of all transfected proteins.

TRAF2DN or TRAF6. Thirty-six hours post transfection cells were harvested under stringent SDS denaturing condition and then subjected to immunoprecipitation for TNFR1 and Western blot for HA-Ub. TRAF2 and TRAF6 did induce a certain level of TNFR1 ubiquitination observed as high-molecular-weight smears (**Figure 4.14**). However, the pattern of TNFR1 ubiquitination was quite different as two strong single bands which are ~10 kD above TNFR1 full-length and CTD respectively were observed and these two bands could be considered as the monoubiquitination form of the TNFR1 full-length and CTD. Monoubiquitination of TNFR1 was also proposed in other studies and was linked to the degradation after its endocytosis through lysosomal pathway (Chin and Horwitz, 2005, 2006). Moreover, over-expression of TRAF2 and TRAF6 appeared to decrease the levels of the TNFR1 full-length and CTD and this effect was dependent on TRAF2 E3 ligase activity. Collectively, TRAF2-mediated ubiquitination may have a total diverse profile comparing to TRAF6-mediated regulation of IL-1R1 and TLR4.

Discussion:

Deficiency of TRAF6 causes severe osteopetrosis, which was observed with defective IL-1, CD40and LPS signalling (Lomaga et al., 1999b; Naito et al., 1999). TRAF6 was reported to be involved in osteoclast maturation and activation through its association with RANK signalling which requires its E3 ligase activity (Armstrong et al., 2002; Lamothe et al., 2007b). However, signalling via IL-1R1 was shown to rescue the osteoclast activation defects caused by the disruption of TRAF6 and RANK interaction (Armstrong et al., 2002) suggesting that IL-1 signalling may also involve in this function. Additionally, TRAF6 is also required for TNF-related apoptosis-inducing ligand (TRAIL) induced osteoclast differentiation (Yen et al., 2012), underlining the importance of TRAF6 in regulating the signalling of TNF superfamily molecules. Deficiency of TRAF6 has also been shown to result in defects in immune and inflammatory responses which are linked to the defective IL-1 and CD40 signalling (Naito et al., 1999; Kobayashi et al., 2009).

TRAF6 is intensively studied as the adaptor protein of IL-1R1/TLR4 signalling complex which facilitates K63-linked polyubiquitination of target protein to trigger recruitment and activation of downstream effector proteins. However, emerging evidences reveal TRAF6 as a regulator of a variety of receptors. For example, TRAF6 mediates polyubiquitination of TrkA which is required for the internalization of TrkA (Geetha et al., 2005). TRAF6-induced K63-linked ubiquitination of Akt is essential for its membrane recruitment and phosphorylation upon ligand stimulation (Yang et al., 2009). Our group previously reported IL-1R1 as a substrate of γ-secretase dependent regulated intramembrane proteolysis and TRAF6 was shown to positively regulate

the cleavage of IL-1R1 (Twomey et al., 2009). Additionally, TRAF6 was shown to induce ubiquitination of IL-1R1, but the function of this modification is to be elucidated.

To investigate the function of TRAF6-mediated ubiquitination of IL-1R1, we attempted to map the sites of IL-1R1 ubiquitination. Ubiquitination of Notch1 receptor was mapped to a single juxtamembrane lysine residue which regulates the γ-secretase cleavage of Notch1 (Gupta-Rossi et al., 2004b). Similarly, we narrowed the potential ubiquitination sites of IL-1R1 into the juxtamembrane domain which contains four lysine residues (**Figure 4.3**). Mutagenesis of single or double lysine residues did not show any dramatic difference in IL-1R1 ubiquitination comparing to the wild-type IL-1R1. However, mutation of lysine 360/378/383 residues resulted in deficient IL-1R1 CTD ubiquitination and reduced CTD level (**Figure 4.7**). The same deficiency was also observed with mutation of two other lysine residues, K527 and K532. These two lysine residues were further mutated and the IL-1R1 K5R mutant showed further reduction in CTD ubiquitination and CTD level (**Figure 4.8**). Additionally we showed that TRAF6 induces polyubiquitination of IL-1R1 and C-terminus fragment is the substrate of TRAF6-mediated ubiquitination (**Figure 4.9**). Similarly, NEMO was also reported to be regulated by TRAF6 through K63-linked polyubiquitination at five different lysine residues, which leads to the activation of IKK and NF-κB (Sebban-Benin et al., 2007b), indicating the possibility of a common pattern in TRAF6-mediated regulation. Additionally, three mutated lysine residues (383,527,532) are localized in the IL-1R1 Toll/IL-1 receptor (TIR) homology domain which mediates receptor interaction with their signal-transduction components. However, these three lysine residues are not revealed in previous studies as

functional motifs of the IL-1R1 TIR domain (Slack et al., 2000). By alignment of the amino acid sequences of five IL-1R family proteins including IL-1R1, IL-1RAcP, IL-18R, ST2 and IL-1Rrp2, we found that K383 and K532 are highly conserved in the TIR domain of these receptors (**Figure 4.15**), suggesting that we may have identified novel functional motifs of IL-1R TIR domain. TRAF6-mediated ubiquitination sites of IL-1R1 intracellular domain could also be narrowed down to these two lysine residues. Studies have shown that IL-1R1 is modified by N-linked glycosylation, which is essential for the optimal IL-1 binding and activation (Mancilla et al., 1992). The diminished higher molecular weight form of full-length IL-1R1 observed in IL-1R1 K5R and K3R mutant (**Figure 4.8**) is probably the glycosylated form of IL-1R1. Therefore, mutagenesis of these lysine residues may not only alter IL-1R1 ubiquitination modification, but also prevent its glycosylation which is critical for the initiation of IL-1 signal transduction. However, the nature of the higher molecular weight form of IL-1R1 requires to be further confirmed.

As mentioned above, TRAF6-mediated polyubiquitination of TrkA is required for its trafficking (Geetha et al., 2005). Similarly we showed that mutagenesis of TRAF6-targeted sites of IL-1R1 caused defects in IL-1R1 membrane distribution (**Figure 4.10**). Furthermore, we showed that IL-1R1 K5R mutant is deficient in IL-1β-induced NF-κB activation (**Figure 4.11**). IL-1R1 was shown to undergo lysosomal degradation after its endocytosis (Brissoni et al., 2006). By inhibiting the lysosomal degradation, we concluded that mutagenesis of TRAF6-regulated sites of IL-1R1 causes deficiency in IL-1R1 ectodomain shedding and diminishes IL-1R1 CTD production, rather than causing rapid IL-1R1 CTD degradation (**Figure 4.12**). Unfortunately, mutagenesis of the five lysine residues in the IL-1R1 C-terminus region did not eliminate the

ubiquitination of full-length IL-1R1. Additional lysine residues in the intracellular domain or even in the extracellular domain may be targeted by TRAF6-mediated ubiquitination. Collectively, TRAF6-mediated polyubiquitination of IL-1R1 intracellular domain is required for its membrane distribution and responsiveness to IL-1β stimulation. Mutating of TRAF6-targeted lysine residues results in defective IL-1R1 ectodomain shedding and dramatic reduction in the IL-1R1 CTD production.

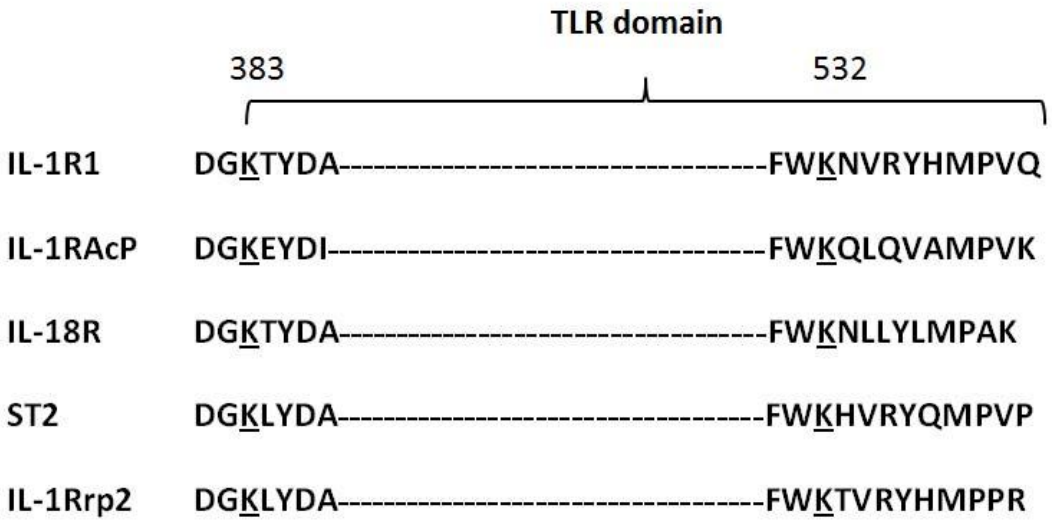

Figure 4.15 Human IL-1R family protein TIR domain alignments. Alignment of amino acid sequences of the TIR domain from human IL-1R1, IL-1RAcP, IL-18R, ST2 and IL-1Rrp2. The highly conserved lysine residues are underlined.

Chapter 5:

GENERAL DISCUSSION

5.1 Summary and Conclusions:

Neurodegenerative disorders are characterized by an abnormal accumulation of neurotoxic macromolecules inside cells and in the extracellular space. In specific regions of the brain, proteins or their fragments undergo alteration in conformation and/or function and form aggregates, such as Aβ plaques and neurofibrillary tangles in AD, Lewy bodies in Parkinson's disease and Inclusion bodies in Amyotrophic lateral sclerosis (ALS) and Huntington's disease (Martin, 1999; Bates, 2003; Dupuis et al., 2006; Wakabayashi et al., 2007). Posttranslational modifications of these neuronal proteins play a critical role in the development of neurodegenerative diseases. One hallmark of AD is the neurofibrillary tangles which consist of paired helical filaments and abnormal hyperphosphorylated microtubule associated protein tau (Iqbal et al., 2009). While normal tau protein stabilizes microtubules and promotes their constitution, glycosylation of tau precedes its abnormal hyperphosphorylation which decreases its turnover and abets the self-assembly into tangles of paired helical or straight filaments, neutrophil threads and dystrophic plaque neuritis (Alonso Adel et al., 2004). The activity of one major phosphatase regulating tau phosphorylation, phosphatase-2A, is down-regulated whereas its inhibitors are over-expressed in the AD brains (Iqbal et al., 2009). Aβ was also shown to be phosphorylated by PKA which promotes Aβ aggregation (Kumar and Walter, 2011). Lipid peroxidation is an early event during the progression of AD and protein bound 4-hydroxy-2-nonenal (HNE) and acrolein have been found to be significantly elevated in AD brain (Lauderback et al., 2001; Lovell et al., 2001; McGrath et al., 2001). Protein-bound HNE alters conformation and function of proteins such as α-enolase, ATP synthase manganese superoxide dismutase, peroxiredoxin VI, dihydropyrimidine-related protein2 (DRP2),

α-tubulin and glutamine synthetase, thus involving in the course of AD including energy metabolism, mitochondrial dysfunction, cytoskeletal integrity, antioxidant defense, protein synthesis, stress response, neuronal communication and excitoxicity (Siems et al., 1996; Subramaniam et al., 1997; Perluigi et al., 2009; Reed et al., 2009; Hellberg et al., 2010). Parkinson's disease pathology is characterized by the presence of Lewy bodies which include α-synuclein, ubiquitin, parkin and synphilin-1 (Wakabayashi et al., 2007). The major constituent of Lewy bodies is α-synuclein. Several studies showed that the majority of α-synuclein within Lewy bodies is phosphorylated at multiple sites in the brains of Parkinson's disease patients and phosphorylation modulates its aggregation and toxicity (Anderson et al., 2006; Mbefo et al., 2010; Oueslati et al., 2010). α-synuclein also undergoes proteolysis and the N-terminus fragment has been shown to promote the fibrillization of the Lewy bodies (Murray et al., 2003; Li et al., 2005). Ubiquitinated α-synuclein has also been detected within the Lewy bodies and ubiquitination of α-synuclein enhances its aggregation and inclusion formation (Lee et al., 2008; Rott et al., 2008; Szargel et al., 2009). Mutations in an important antioxidant, superoxide dismutase 1 (SOD1), contribute to the onset of ALS (Rosen, 1993). Activation of the SOD1 involves several posttranslational modification including copper and zinc binding and formation of intramolecular disulfide bonds (Furukawa and O'Halloran, 2006). Mutant of SOD1 has been shown to exhibit increased affinity for copper and zinc-deficient SOD1 was shown to be superoxidative and therefore rapidly oxidize many intracellular components (Crow et al., 1997; Watanabe et al., 2007). Additionally, wild-type SOD1 was shown able to acquire toxic properties similar to familial ALS-linked mutant SOD1 by hyper-oxidation (Guareschi et al., 2012).

Huntington's disease emerges as a consequence of a polyglutamine repeat expansion in the huntingtin gene (Ashley and Warren, 1995). The abnormal huntingtin protein undergoes proteolysis into fragments and these fragments are modified by ubiquitin. However, these fragments targeted by proteasome are not efficiently degraded, therefore leading to accumulation of aggregates forming neuronal inclusion bodies (Bates, 2003; Wang and Lashuel, 2012).

A number of studies have reported that presenilins play an important role in synaptic function in the adult cerebral cortex (Ho and Shen, 2011). PS1 was found to associate with the postsynaptic N-methyl-D-aspartic acid receptor (NMDAR) and is thought to function at synapses by facilitating the proper synaptic delivery and localization of NMDARs (Saura et al., 2004). *In vivo* studies revealed essential roles of presenilins in synaptic plasticity, learning and memory and neuronal survival in the adult cerebral cortex. PS1 conditional knockout mice exhibited mild cognitive deficits in long-term spatial memory which appeared to be independent of the Notch signaling pathway (Yu et al., 2001). Conditional double knockout of PS1 and PS2 in the postnatal forebrain revealed severe deficits in hippocampal learning and memory as well as synaptic plasticity impairments (Saura et al., 2004). Inactivation of presenilins also resulted in delayed apoptosis and low percentage of apoptotic cell death (Wines-Samuelson et al., 2010). An Ephrin receptor family member, EphA4, was identified as a substrate of γ-secretase and the EphA4 intracellular domain increases the number of dendritic spines (Inoue et al., 2009). Age-dependent *in vivo* accumulation of APP C-terminal fragments was observed at presynaptic terminals after inhibition of γ-secretase activity by PS1 inactivation, indicating the existing role of APP signalling in neuronal functions (Saura et al., 2005). γ-secretase-mediated proteolysis of APP is

very similar to that of Notch. Elevated AICD levels have been reported in the AD brain (Ghosal et al., 2009). AICD was also reported to form complex with the nuclear adaptor protein Fe65 and the histone acetyltransferase Tip60 and translocate to the nucleus (Cao and Sudhof, 2001; Slomnicki and Lesniak, 2008). Transgenic mice overexpressing AICD and Fe65 showed abnormal activity of GSK3β which leads to hyperphosphorylation and aggregation of tau (Ryan and Pimplikar, 2005). Phosphorylation of the APP cytoplasmic domain by JNK3, the neuron-specific JNK protein, disrupts the interaction between AICD and Fe65 during neuron differentiation (Kimberly et al., 2005). Additionally, AICD expression was shown to induce neuron-specific apoptosis (Nakayama et al., 2008). AICD was also shown to regulate the expression of over 600 genes and some of them are related to cell death (Ohkawara et al., 2011).

Intensive studies have focused on the role of presenilins as the catalytic core of γ-secretase complex. Accordingly, efforts have been put on understanding the regulated intramembrane proteolysis of APP, the subsequent generation and deposits of Aβ and the selective inhibition of γ-secretase activity as potential treatment for AD patients (Jakob-Roetne and Jacobsen, 2009; Wolfe, 2009). However, γ-secretase-independent functions of presenilins have also been revealed, including their role in calcium signalling, cell adhesion, protein trafficking and apoptosis (Hass et al., 2008; Coen and Annaert, 2010). Non-proteolytic functions of presenilins are still expanding. Presenilins are recently reported to be necessary for the efficient lysosomal-autophagic degradation (Neely et al., 2011). More importantly, a number of familiar AD mutants of presenilin have been reported to alter the γ-secretase-independent functions of presenilins (Nelson et al., 2007; Lee et

al., 2010), which may provide a different insight into the pathogenesis of AD. Presenilin involves in the regulation of signal transduction via either cleavage of γ-secretase substrates or interacting with signalling protein such as β-catenin (Xia et al., 2001). Presenilin interacts with at least 60 different proteins, one among them is TRAF6 (McCarthy et al., 2009a). TRAF6 is an adaptor protein essential for the receptor complex of two γ-secretase substrates, IL-1R1 and $p75^{NTR}$. However, by studying the interaction between TRAF6 and presenilin, we revealed that TRAF6 does not affect γ-secretase activity or apart from presenilin, does not appear to regulate any other component of γ-secretase complexes. We showed that TRAF6 stabilizes full-length presenilins by inducing K63-linked polyubiquitination. Furthermore we showed that regulation by TRAF6 may involve in the full-length function of presenilin as passive ER Ca^{2+} leak channels.

IL-1 is a master cytokine of local and systemic inflammation and neutralization of IL-1β results in a rapid and sustained reduction in disease severity. IL-1α precursor is constitutively present in the cells of healthy individuals whereas IL-1β is a product of a limited number of cells including blood monocytes, tissue macrophages and dendritic cells and activation of IL-1β precursor requires intracellular cleavage by caspase 1 (Agostini et al., 2004; Martinon et al., 2009). Caspase-1 existing in tissue macrophages and dendritic cells is inactive and requires a specialized group of intracellular proteins termed the “inflammasome” for its activation (Agostini et al., 2004). However, caspase-1 is present in an active state in circulating human blood monocytes and highly metastatic human melanoma cells (Netea et al., 2009; Okamoto et al., 2010). Higher level of IL-1β was detected in blood monocytes from patients with autoinflammatory disease than the cells from healthy individuals

(Gattorno et al., 2008; Colina et al., 2010). An increase in the secretion of active IL-1β is observed in monocytes from patients with a gain-of-function mutation in a gene originally called cold-induced autoinflammatory syndrome-1 (Hoffman et al., 2001). This mutation results in a single amino acid change in an intracellular protein named nucleotide-binding domain and leucine-rich repeat-containing protein 3 (NLRP3), which associates with procaspase-1 and other intracellular proteins to form the "inflammasome" (Agostini et al., 2004). However, approximately one-half of the patients with classic symptoms and biochemical markers of Cryopyrin-associated periodic syndromes (CAPSs), familial Mediterranean fever and other autoinflammatory diseases do not have mutations. Caspase-1-independent activation of IL-1β has also been reported, for example, irritant-induced inflammation in muscle tissue, sartilage destruction in joints and urate crystal-induced inflammation are IL-1β dependent but caspase-1 independent (Fantuzzi et al., 1997; Guma et al., 2009; Joosten et al., 2009). Moreover, one of the neutrophil proteases, proteinase-3, cleaves the inactive IL-1β close to caspase-1 cleavage site and releases active IL-1β (Fantuzzi et al., 1997; Joosten et al., 2009). In autoimmune responses, IL-1β has been shown to increase the expansion of naive and memory CD4 T cells and is required for red blood cell antibody productions (Ben-Sasson et al., 2009). IL-1β also plays a pivotal role in the differentiation of T cells in to Th17 cells and mice deficient for IL-1R1 or IRAK1 do not develop Th17 cells (Matsuki et al., 2006; Chung et al., 2009; Gulen et al., 2010; Joosten, 2010). Neuroinflammation has been recognized as a fundamental response to not only acute injury, but also to chronic neurodegenerative disease as severity of neuroinflammatory response has been observed alongside the course of AD (Sheng et al., 1997a, b). Elevations of IL-1β have

been detected in the brains of aged AD mouse models and plaque associated microglia (Benzing et al., 1999; Lim et al., 2000). Additionally, IL-1 has been shown to increase APP mRNA expression, translation and its γ-secretase cleavage (Goldgaber et al., 1989; Gray and Patel, 1993; Brugg et al., 1995; Rogers et al., 1999; Liao et al., 2004). Therapeutic strategies for autoinflammatory and autoimmune diseases have been focused on the blockade of IL-1 signalling. Competing for IL-1R ligand-binding sites was achieved by applying naturally occurring or chimeric IL-1R antagonist (IL-1Ra) and IL-1R-blocking antibody. IL-1 could also be neutralized by soluble IL-1 decoy receptors and anti-IL-1 antibodies (Dinarello, 2011; Dinarello et al., 2012).

In addition to IL-1R1, over one hundred type I integral membrane proteins have been identified as substrates for γ-secretase-dependent proteolysis (McCarthy et al., 2009a). Regulated intramembrane proteolysis by γ-secretase therefore involves in the regulation of multiple cellular events including differentiation (Shih Ie and Wang, 2007), gene transcription (May et al., 2002; Murakami et al., 2003; Kinoshita et al., 2006), cell adhesion (Ferber et al., 2008; Waschbusch et al., 2009), protein turnover (Kopan and Ilagan, 2004) and immune response (Carey et al., 2007; McCarthy et al., 2009b). A common feature of all the γ-secretase substrates is that all substrates release their ectodomain after proteolysis in their extracellular domain and ectodomain shedding is required for the subsequent γ-secretase cleavage. However, relatively little is known about how γ-secretase cleavage is regulated, where the cleavage occurs and what modification of the substrate is involved in this proteolysis. Palmitoylation, ubiquitination and phosphorylation of the γ-secretase substrates have been shown to regulation the γ-secretase cleavage of its substrates (Gupta-Rossi et al., 2004a; Takahashi et al., 2008; Underwood and Coulson, 2008). IL-1R1

was previously reported to be ubiquitinated by TRAF6 and this regulation involves in the proteolysis and signalling transduction of this receptor (Twomey et al., 2009). In this study, by further characterizing TRAF6-mediated ubiquitination of IL-1R1, we found that five lysine residues in IL-1R1 C-terminus are targeted by TRAF6-mediated polyubiquitination. Moreover, mutagenesis of these five lysine residues reduces the cell surface localization of IL-1R1, attenuates the responsiveness to IL-1β stimulation and diminishes the production of IL-1R1 CTD, suggesting that TRAF6-mediated ubiquitination is essential for the distribution and signalling transduction of IL-1R1.

5.2 Future Perspectives:

5.2.1 Map ubiquitination sites of presenilins and investigate the functions of TRAF6-mediated ubiquitination of presenilins

We have performed site-directed mutagenesis and *in vitro* ubiquitination assay of peptide arrays to map the sites of PS1 ubiquitination sites. Single or double lysine mutations of PS1 did not reveal any potential sites for PS1 ubiquitination suggesting that multiple lysine residues may be involved in this modification. *In vitro* ubiquitination assay could still be optimized by figuring out if TRAF6K124R mutation has E3 ligase activity *in vitro* or by distinguishing ubiquitination of substrates from TRAF6 autoubiquitination. Then the presenilin peptide arrays could still be used to determine the ubiquitination sites. Additionally, our group is generating new constructs to be used in mass spectrometry analysis to identify the ubiquitination sites of certain candidate proteins.

Upon identifying the ubiquitination sites of presenilin, we could mutate these residues and test the property of these mutations in terms of protein turnover, TRAF6-mediated ubiquitination and γ-secretase activity. Knock-out of TRAF6 has been shown to attenuate the ER calcium signalling and we assume that destabilized full-length presenilins cause this deficiency. By obtaining presenilins mutants that are deficient in TRAF6-mediated ubiquitination, we could verify that if TRAF6-mediated ubiquitination of presenilin involves in the regulation of the ER Ca^{2+} leak function. Furthermore, by immunohistochemistry approaches we could determine in which subcellular compartment this regulation occurs and the correlation between presenilin ubiquitination and proteasomal degradation. These experiments would provide novel insights into how full-length presenilins are regulated and help understanding the functions of full-length presenilins. As disrupted function of full-length presenilin has been linked to familial AD mutants (Nelson et al., 2007), our study will provide understanding of the relevance between γ-secretase independent presenilin functions and AD pathogenesis.

In addition to TRAF6, other TRAF family members have also been shown to stabilize presenilins and induce ubiquitination. However TRAF2DN mutant has also been shown to enhance presenilin levels. By further characterizing presenilin ubiquitination mediated by other TRAFs, we would find out if other TRAF proteins induce presenilin ubiquitination through their E3 ligase activity or only enhance this modification as adaptor proteins. These experiments would reveal the redundancy among TRAF family members and help to understand regulation of full-length presenilins by TRAF family proteins.

5.2.2 Further characterize the role of TRAF6-mediated ubiquitination in IL-1R1 protein turnover, trafficking and regulated intramembrane proteolysis.

One study reported that lysine residues are preferentially ubiquitinated when they are localized next to certain residues which could be phosphorylated or are polar uncharged or negatively charged (Catic et al., 2004). Thirteen out of twenty-one lysine residues in the intracellular domain of IL-1R1 fit into these profiles and 3 of them (K383, 527, 532) are identified in this study as TRAF6-mediated ubiquitination sites of IL-1R1. K383 and K532 are also identified as highly conserved motifs in the TIR domain of IL-1R family members. Mutagenesis of these five lysine residues reduces the membrane localization of IL-1R1, attenuates the responsiveness to IL-1β stimulation and diminishes the production of IL-1R1 CTD. Further characterization should be able to show if TRAF6-mediated ubiquitination is required for internalization or the ectodomain shedding and proteolysis of the IL-1R1 receptor and thus find out the cause for deficient IL-1R1 CTD production. By subcellular fractionation and immunohistochemistry, it is also possible to determine that when the IL-1R1 K5R mutant is deficient in plasma membrane localization, in what other subcellular compartment does it accumulate and does the mutant have alternative trafficking or degradative pathway. Moreover, requirement of TRAF6-mediated ubiquitination for IL-1R1 signalling transduction could be investigated in terms of ligand binding, signalling complex assembly and downstream signalling protein recruitment. Further studies would generate intriguing insight into the regulation of IL-1R1 signalling and distinguish between γ-secretase cleavage dependent and independent IL-1R1 signalling.

Although IL-1R1 K5R mutant is deficient in membrane localization, ubiquitination of full-length receptor mediated by TRAF6 was not affected comparing to wild-type IL-1R1 and full-length mutant was still stabilized by TRAF6. This observation led us to the speculation that TRAF6 may associate with IL-1R1 not only in the membrane signalling complex, but also in other compartments. As mentioned above, mass spectrometry analysis is going to be applied for mapping other ubiquitination sites of IL-1R1. Identifying additional ubiquitination sites in IL-1R1 intracellular domain or even in the ectodomain may lead us to understand the mechanism how the full-length IL-1R1 is modified and transported and why TRAF6 enhances the level of IL-1R1 full-length protein. As blockade of IL-1 signalling is considered as therapeutic strategy for some autoinflammatory and autoimmune diseases, our discovery of important residues for IL-1R1 signalling transduction may provide alternative approach for the treatment of IL-1 related diseases.

TLR4 and TNFR1 were also shown to be regulated by TRAF6 and TRAF2 respectively through ubiquitination. TLR4 seemed to adhere to similar pattern of regulation by TRAF6-mediated ubiquitination as IL-1R1 where TRAF6 induces ubiquitination and enhances cellular level of the receptor. Upon mapping the sites of TLR4 ubiquitination, mechanism of this regulation should be revealed by experiments that have been performed with IL-1R1. However, TRAF2-mediated ubiquitination of TNFR1 seemed to play a total diverse role where TNFR1 showed a different ubiquitination pattern and was destabilized by TRAF2 and TRAF6. TRAF2 and TRAF6 have both been report to negatively regulate TNFR1 activity (Nguyen et al., 1999; Funakoshi-Tago et al., 2009). Therefore, further characterization of TRAF-mediated

ubiquitination of TNFR1 may provide novel understanding of TNF signalling and the diversity of TRAF family mediated regulation. Additionally, study of the γ-secretase dependent cleavage of its substrate will generate novel knowledge about the regulated intramembrane proteolysis of these type I integral membrane proteins and the functional significance of their intracellular domain generated by γ-secretase cleavage.

Bibliography

Abbott DW, Yang Y, Hutti JE, Madhavarapu S, Kelliher MA, Cantley LC (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. Mol Cell Biol 27:6012-6025.

Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity 20:319-325.

Alonso Adel C, Mederlyova A, Novak M, Grundke-Iqbal I, Iqbal K (2004) Promotion of hyperphosphorylation by frontotemporal dementia tau mutations. The Journal of biological chemistry 279:34873-34881.

Anderson JP, Walker DE, Goldstein JM, de Laat R, Banducci K, Caccavello RJ, Barbour R, Huang J, Kling K, Lee M, Diep L, Keim PS, Shen X, Chataway T, Schlossmacher MG, Seubert P, Schenk D, Sinha S, Gai WP, Chilcote TJ (2006) Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. The Journal of biological chemistry 281:29739-29752.

Annaert WG, Esselens C, Baert V, Boeve C, Snellings G, Cupers P, Craessaerts K, De Strooper B (2001) Interaction with telencephalin and the amyloid precursor protein predicts a ring structure for presenilins. Neuron 32:579-589.

Aoyagi N, Uemura K, Kuzuya A, Kihara T, Kawamata J, Shimohama S, Kinoshita A, Takahashi R (2010) PI3K inhibition causes the accumulation of ubiquitinated presenilin 1 without affecting the proteasome activity. Biochemical and biophysical research communications 391:1240-1245.

Area-Gomez E, de Groof AJ, Boldogh I, Bird TD, Gibson GE, Koehler CM, Yu WH, Duff KE, Yaffe MP, Pon LA, Schon EA (2009) Presenilins are enriched in endoplasmic reticulum membranes associated with mitochondria. The American journal of pathology 175:1810-1816.

Armstrong AP, Tometsko ME, Glaccum M, Sutherland CL, Cosman D, Dougall WC (2002) A RANK/TRAF6-dependent signal transduction pathway is essential for osteoclast cytoskeletal organization and resorptive function. The Journal of biological chemistry 277:44347-44356.

Ashley CT, Jr., Warren ST (1995) Trinucleotide repeat expansion and human disease. Annu Rev Genet 29:703-728.

Au PY, Yeh WC (2007) Physiological roles and mechanisms of signaling by TRAF2 and TRAF5. Adv Exp Med Biol 597:32-47.

Aveleira C, Castilho A, Baptista F, Simoes N, Fernandes C, Leal E, Ambrosio AF (2010) High glucose and interleukin-1beta downregulate interleukin-1 type I receptor (IL-1RI) in retinal endothelial cells by enhancing its degradation by a lysosome-dependent mechanism. Cytokine 49:279-286.

Bai S, Kopan R, Zou W, Hilton MJ, Ong CT, Long F, Ross FP, Teitelbaum SL (2008) NOTCH1 regulates osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblast lineage cells. The Journal of biological chemistry 283:6509-6518.

Bai S, Kitaura H, Zhao H, Chen J, Muller JM, Schule R, Darnay B, Novack DV, Ross FP, Teitelbaum SL (2005) FHL2 inhibits the activated osteoclast in a TRAF6-dependent manner. J Clin Invest 115:2742-2751.

Baki L, Neve RL, Shao Z, Shioi J, Georgakopoulos A, Robakis NK (2008) Wild-type but not FAD mutant presenilin-1 prevents neuronal degeneration by promoting phosphatidylinositol 3-kinase neuroprotective signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:483-490.

Baki L, Marambaud P, Efthimiopoulos S, Georgakopoulos A, Wen P, Cui W, Shioi J, Koo E, Ozawa M, Friedrich VL, Jr., Robakis NK (2001) Presenilin-1 binds cytoplasmic epithelial cadherin, inhibits cadherin/p120 association, and regulates stability and function of the cadherin/catenin adhesion complex. Proceedings of the National Academy of Sciences of the United States of America 98:2381-2386.

Barakat A, Mercer B, Cooper E, Chung HM (2009) Examining requirement for formation of functional Presenilin proteins and their processing events in vivo. Genesis.
Bates G (2003) Huntingtin aggregation and toxicity in Huntington's disease. Lancet 361:1642-1644.
Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, Dinarello CA, Paul WE (2009) IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proceedings of the National Academy of Sciences of the United States of America 106:7119-7124.
Bentahir M, Nyabi O, Verhamme J, Tolia A, Horre K, Wiltfang J, Esselmann H, De Strooper B (2006) Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. Journal of neurochemistry 96:732-742.
Benzing WC, Wujek JR, Ward EK, Shaffer D, Ashe KH, Younkin SG, Brunden KR (1999) Evidence for glial-mediated inflammation in aged APP(SW) transgenic mice. Neurobiology of aging 20:581-589.
Berridge MJ (2010) Calcium hypothesis of Alzheimer's disease. Pflugers Arch 459:441-449.
Bezprozvanny I (2012) Presenilins: a novel link between intracellular calcium signaling and lysosomal function? The Journal of cell biology 198:7-10.
Biederer T, Volkwein C, Sommer T (1997) Role of Cue1p in ubiquitination and degradation at the ER surface. Science 278:1806-1809.
Bird TA, Woodward A, Jackson JL, Dower SK, Sims JE (1991) Phorbol ester induces phosphorylation of the 80 kilodalton murine interleukin 1 receptor at a single threonine residue. Biochemical and biophysical research communications 177:61-67.
Blonska M, You Y, Geleziunas R, Lin X (2004) Restoration of NF-kappaB activation by tumor necrosis factor alpha receptor complex-targeted MEKK3 in receptor-interacting protein-deficient cells. Molecular and cellular biology 24:10757-10765.
Boulton ME, Cai J, Grant MB (2008) gamma-Secretase: a multifaceted regulator of angiogenesis. J Cell Mol Med 12:781-795.
Bradley JR, Pober JS (2001) Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene 20:6482-6491.
Brikos C, Wait R, Begum S, O'Neill LA, Saklatvala J (2007) Mass spectrometric analysis of the endogenous type I interleukin-1 (IL-1) receptor signaling complex formed after IL-1 binding identifies IL-1RAcP, MyD88, and IRAK-4 as the stable components. Mol Cell Proteomics 6:1551-1559.
Brissoni B, Agostini L, Kropf M, Martinon F, Swoboda V, Lippens S, Everett H, Aebi N, Janssens S, Meylan E, Felberbaum-Corti M, Hirling H, Gruenberg J, Tschopp J, Burns K (2006) Intracellular trafficking of interleukin-1 receptor I requires Tollip. Current biology : CB 16:2265-2270.
Brugg B, Dubreuil YL, Huber G, Wollman EE, Delhaye-Bouchaud N, Mariani J (1995) Inflammatory processes induce beta-amyloid precursor protein changes in mouse brain. Proceedings of the National Academy of Sciences of the United States of America 92:3032-3035.
Brunello L, Zampese E, Florean C, Pozzan T, Pizzo P, Fasolato C (2009) Presenilin-2 dampens intracellular Ca2+ stores by increasing Ca2+ leakage and reducing Ca2+ uptake. Journal of cellular and molecular medicine 13:3358-3369.
Brunkan AL, Martinez M, Walker ES, Goate AM (2005) Presenilin endoproteolysis is an intramolecular cleavage. Mol Cell Neurosci 29:65-73.
Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S, Lacey DL (1999) The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. The Journal of cell biology 145:527-538.
Burns K, Clatworthy J, Martin L, Martinon F, Plumpton C, Maschera B, Lewis A, Ray K, Tschopp J, Volpe F (2000) Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nature cell biology 2:346-351.

Butler MP, Hanly JA, Moynagh PN (2007) Kinase-active interleukin-1 receptor-associated kinases promote polyubiquitination and degradation of the Pellino family: direct evidence for PELLINO proteins being ubiquitin-protein isopeptide ligases. The Journal of biological chemistry 282:29729-29737.

Cao X, Sudhof TC (2001) A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293:115-120.

Carey BW, Kim DY, Kovacs DM (2007) Presenilin/gamma-secretase and alpha-secretase-like peptidases cleave human MHC Class I proteins. Biochem J 401:121-127.

Carmody RJ, Ruan Q, Palmer S, Hilliard B, Chen YH (2007) Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. Science 317:675-678.

Carpenter G, Red Brewer M (2009) EpCAM: another surface-to-nucleus missile. Cancer Cell 15:165-166.

Catic A, Collins C, Church GM, Ploegh HL (2004) Preferred in vivo ubiquitination sites. Bioinformatics 20:3302-3307.

Chen B, Mariano J, Tsai YC, Chan AH, Cohen M, Weissman AM (2006) The activity of a human endoplasmic reticulum-associated degradation E3, gp78, requires its Cue domain, RING finger, and an E2-binding site. Proc Natl Acad Sci U S A 103:341-346.

Chen Q, Chen T, Li W, Zhang W, Zhu J, Li Y, Huang Y, Shen Y, Yu C (2012) Mycoepoxydiene Inhibits Lipopolysaccharide-Induced Inflammatory Responses through the of TRAF6 Polyubiquitination. PLoS ONE 7:e44890.

Chen ZJ, Sun LJ (2009) Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell 33:275-286.

Cheng H, Addona T, Keshishian H, Dahlstrand E, Lu C, Dorsch M, Li Z, Wang A, Ocain TD, Li P, Parsons TF, Jaffee B, Xu Y (2007) Regulation of IRAK-4 kinase activity via autophosphorylation within its activation loop. Biochemical and biophysical research communications 352:609-616.

Chin YR, Horwitz MS (2005) Mechanism for removal of tumor necrosis factor receptor 1 from the cell surface by the adenovirus RIDalpha/beta complex. J Virol 79:13606-13617.

Chin YR, Horwitz MS (2006) Adenovirus RID complex enhances degradation of internalized tumour necrosis factor receptor 1 without affecting its rate of endocytosis. The Journal of general virology 87:3161-3167.

Chung JY, Park YC, Ye H, Wu H (2002a) All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. Journal of cell science 115:679-688.

Chung JY, Park YC, Ye H, Wu H (2002b) All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci 115:679-688.

Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, Dong C (2009) Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity 30:576-587.

Citron M, Teplow DB, Selkoe DJ (1995) Generation of amyloid beta protein from its precursor is sequence specific. Neuron 14:661-670.

Citron M et al. (1997) Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice. Nature medicine 3:67-72.

Coen K, Annaert W (2010) Presenilins: how much more than gamma-secretase?! Biochemical Society transactions 38:1474-1478.

Coen K, Flannagan RS, Baron S, Carraro-Lacroix LR, Wang D, Vermeire W, Michiels C, Munck S, Baert V, Sugita S, Wuytack F, Hiesinger PR, Grinstein S, Annaert W (2012) Lysosomal calcium homeostasis defects, not proton pump defects, cause endo-lysosomal dysfunction in PSEN-deficient cells. The Journal of cell biology 198:23-35.

Colina M, Pizzirani C, Khodeir M, Falzoni S, Bruschi M, Trotta F, Di Virgilio F (2010) Dysregulation of P2X7 receptor-inflammasome axis in SAPHO syndrome: successful treatment with anakinra. Rheumatology (Oxford) 49:1416-1418.

Conze DB, Wu CJ, Thomas JA, Landstrom A, Ashwell JD (2008) Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation. Molecular and cellular biology 28:3538-3547.

Cox RT, McEwen DG, Myster DL, Duronio RJ, Loureiro J, Peifer M (2000) A screen for mutations that suppress the phenotype of Drosophila armadillo, the beta-catenin homolog. Genetics 155:1725-1740.

Crow JP, Sampson JB, Zhuang Y, Thompson JA, Beckman JS (1997) Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite. Journal of neurochemistry 69:1936-1944.

Cui S, Xiong F, Hong Y, Jung JU, Li XS, Liu JZ, Yan R, Mei L, Feng X, Xiong WC (2011) APPswe/Abeta regulation of osteoclast activation and RAGE expression in an age-dependent manner. J Bone Miner Res 26:1084-1098.

da Silva Correia J, Ulevitch RJ (2002) MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. The Journal of biological chemistry 277:1845-1854.

Darnay BG, Ni J, Moore PA, Aggarwal BB (1999) Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. The Journal of biological chemistry 274:7724-7731.

Das HK, Tchedre K, Mueller B (2012) Repression of transcription of presenilin-1 inhibits gamma-secretase independent ER Ca(2)(+) leak that is impaired by FAD mutations. Journal of neurochemistry 122:487-500.

Davies BA, Topp JD, Sfeir AJ, Katzmann DJ, Carney DS, Tall GG, Friedberg AS, Deng L, Chen Z, Horazdovsky BF (2003) Vps9p CUE domain ubiquitin binding is required for efficient endocytic protein traffic. J Biol Chem 278:19826-19833.

Davies CC, Mak TW, Young LS, Eliopoulos AG (2005) TRAF6 is required for TRAF2-dependent CD40 signal transduction in nonhemopoietic cells. Molecular and cellular biology 25:9806-9819.

De Strooper B (2003) Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex. Neuron 38:9-12.

De Strooper B (2007) Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. EMBO Rep 8:141-146.

De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. Nature 391:387-390.

Dehvari N, Cedazo-Minguez A, Isacsson O, Nilsson T, Winblad B, Karlstrom H, Benedikz E, Cowburn RF (2007) Presenilin dependence of phospholipase C and protein kinase C signaling. J Neurochem 102:848-857.

Dejaegere T, Serneels L, Schafer MK, Van Biervliet J, Horre K, Depboylu C, Alvarez-Fischer D, Herreman A, Willem M, Haass C, Hoglinger GU, D'Hooge R, De Strooper B (2008) Deficiency of Aph1B/C-gamma-secretase disturbs Nrg1 cleavage and sensorimotor gating that can be reversed with antipsychotic treatment. Proc Natl Acad Sci U S A 105:9775-9780.

Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103:351-361.

Dinarello CA (2011) Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 117:3720-3732.
Dinarello CA, Simon A, van der Meer JW (2012) Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov 11:633-652.
Dobrowolski R, Vick P, Ploper D, Gumper I, Snitkin H, Sabatini DD, De Robertis EM (2012) Presenilin Deficiency or Lysosomal Inhibition Enhances Wnt Signaling through Relocalization of GSK3 to the Late-Endosomal Compartment. Cell Rep 2:1316-1328.
Dower SK, Wignall JM, Schooley K, McMahan CJ, Jackson JL, Prickett KS, Lupton S, Cosman D, Sims JE (1989) Retention of ligand binding activity by the extracellular domain of the IL-1 receptor. Journal of immunology 142:4314-4320.
Dumanchin C, Czech C, Campion D, Cuif MH, Poyot T, Martin C, Charbonnier F, Goud B, Pradier L, Frebourg T (1999) Presenilins interact with Rab11, a small GTPase involved in the regulation of vesicular transport. Human molecular genetics 8:1263-1269.
Dupuis L, Gonzalez de Aguilar JL, Echaniz-Laguna A, Loeffler JP (2006) Mitochondrial dysfunction in amyotrophic lateral sclerosis also affects skeletal muscle. Muscle Nerve 34:253-254.
Edbauer D, Winkler E, Regula JT, Pesold B, Steiner H, Haass C (2003) Reconstitution of gamma-secretase activity. Nat Cell Biol 5:486-488.
Ellison JM, Tremblay M, Hostager B (2006) Characterizing the Roles of Traf2 and Traf6 in Cd40 Signaling. Journal of Pediatric Gastroenterology and Nutrition 43:E15.
Elzinga BM, Twomey C, Powell JC, Harte F, McCarthy JV (2009a) Interleukin-1 receptor type 1 is a substrate for gamma-secretase-dependent regulated intramembrane proteolysis. J Biol Chem 284:1394-1409.
Elzinga BM, Twomey C, Powell JC, Harte F, McCarthy JV (2009b) Interleukin-1 receptor type 1 is a substrate for gamma-secretase-dependent regulated intramembrane proteolysis. The Journal of biological chemistry 284:1394-1409.
Esler WP, Kimberly WT, Ostaszewski BL, Ye W, Diehl TS, Selkoe DJ, Wolfe MS (2002) Activity-dependent isolation of the presenilin- gamma -secretase complex reveals nicastrin and a gamma substrate. Proc Natl Acad Sci U S A 99:2720-2725.
Esler WP, Kimberly WT, Ostaszewski BL, Diehl TS, Moore CL, Tsai JY, Rahmati T, Xia W, Selkoe DJ, Wolfe MS (2000) Transition-state analogue inhibitors of gamma-secretase bind directly to presenilin-1. Nature cell biology 2:428-434.
Esselens C, Oorschot V, Baert V, Raemaekers T, Spittaels K, Serneels L, Zheng H, Saftig P, De Strooper B, Klumperman J, Annaert W (2004) Presenilin 1 mediates the turnover of telencephalin in hippocampal neurons via an autophagic degradative pathway. The Journal of cell biology 166:1041-1054.
Etcheberrigaray R, Hirashima N, Nee L, Prince J, Govoni S, Racchi M, Tanzi RE, Alkon DL (1998) Calcium responses in fibroblasts from asymptomatic members of Alzheimer's disease families. Neurobiology of disease 5:37-45.
Fantuzzi G, Ku G, Harding MW, Livingston DJ, Sipe JD, Kuida K, Flavell RA, Dinarello CA (1997) Response to local inflammation of IL-1 beta-converting enzyme- deficient mice. Journal of immunology 158:1818-1824.
Ferber EC, Kajita M, Wadlow A, Tobiansky L, Niessen C, Ariga H, Daniel J, Fujita Y (2008) A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus. J Biol Chem 283:12691-12700.
Fleck D, Garratt AN, Haass C, Willem M (2011) BACE1 Dependent Neuregulin Proteolysis. Current Alzheimer research.
Fluhrer R, Friedlein A, Haass C, Walter J (2004) Phosphorylation of presenilin 1 at the caspase recognition site regulates its proteolytic processing and the progression of apoptosis. J Biol Chem 279:1585-1593.

Ford DL, Monteiro MJ (2007) Studies of the role of ubiquitination in the interaction of ubiquilin with the loop and carboxyl terminal regions of presenilin-2. Biochemistry 46:8827-8837.

Fraering PC, LaVoie MJ, Ye W, Ostaszewski BL, Kimberly WT, Selkoe DJ, Wolfe MS (2004a) Detergent-dependent dissociation of active gamma-secretase reveals an interaction between Pen-2 and PS1-NTF and offers a model for subunit organization within the complex. Biochemistry 43:323-333.

Fraering PC, Ye W, Strub JM, Dolios G, LaVoie MJ, Ostaszewski BL, van Dorsselaer A, Wang R, Selkoe DJ, Wolfe MS (2004b) Purification and characterization of the human gamma-secretase complex. Biochemistry 43:9774-9789.

Fraser PE, Levesque G, Yu G, Mills LR, Thirlwell J, Frantseva M, Gandy SE, Seeger M, Carlen PL, St George-Hyslop P (1998) Presenilin 1 is actively degraded by the 26S proteasome. Neurobiology of aging 19:S19-21.

Fukumori A, Fluhrer R, Steiner H, Haass C (2010) Three-amino acid spacing of presenilin endoproteolysis suggests a general stepwise cleavage of gamma-secretase-mediated intramembrane proteolysis. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:7853-7862.

Fukumori A, Okochi M, Tagami S, Jiang J, Itoh N, Nakayama T, Yanagida K, Ishizuka-Katsura Y, Morihara T, Kamino K, Tanaka T, Kudo T, Tanii H, Ikuta A, Haass C, Takeda M (2006) Presenilin-dependent gamma-secretase on plasma membrane and endosomes is functionally distinct. Biochemistry 45:4907-4914.

Fukushima H, Nakao A, Okamoto F, Shin M, Kajiya H, Sakano S, Bigas A, Jimi E, Okabe K (2008) The association of Notch2 and NF-kappaB accelerates RANKL-induced osteoclastogenesis. Molecular and cellular biology 28:6402-6412.

Funakoshi-Tago M, Kamada N, Shimizu T, Hashiguchi Y, Tago K, Sonoda Y, Kasahara T (2009) TRAF6 negatively regulates TNFalpha-induced NF-kappaB activation. Cytokine 45:72-79.

Furukawa Y, O'Halloran TV (2006) Posttranslational modifications in Cu,Zn-superoxide dismutase and mutations associated with amyotrophic lateral sclerosis. Antioxid Redox Signal 8:847-867.

Galibert L, Tometsko ME, Anderson DM, Cosman D, Dougall WC (1998) The involvement of multiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. The Journal of biological chemistry 273:34120-34127.

Gallis B, Prickett KS, Jackson J, Slack J, Schooley K, Sims JE, Dower SK (1989) IL-1 induces rapid phosphorylation of the IL-1 receptor. Journal of immunology 143:3235-3240.

Gattorno M, Piccini A, Lasiglie D, Tassi S, Brisca G, Carta S, Delfino L, Ferlito F, Pelagatti MA, Caroli F, Buoncompagni A, Viola S, Loy A, Sironi M, Vecchi A, Ravelli A, Martini A, Rubartelli A (2008) The pattern of response to anti-interleukin-1 treatment distinguishes two subsets of patients with systemic-onset juvenile idiopathic arthritis. Arthritis Rheum 58:1505-1515.

Geetha T, Jiang J, Wooten MW (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Molecular cell 20:301-312.

Ghosal K, Vogt DL, Liang M, Shen Y, Lamb BT, Pimplikar SW (2009) Alzheimer's disease-like pathological features in transgenic mice expressing the APP intracellular domain. Proceedings of the National Academy of Sciences of the United States of America 106:18367-18372.

Ghosh AK, Hong L, Tang J (2002) beta-Secretase as a Therapeutic Target for Inhibitor Drugs. Curr Med Chem 9:1135-1144.

Ghosh AK, Kumaragurubaran N, Tang J (2005) Recent developments of structure based beta-secretase inhibitors for Alzheimer's disease. Curr Top Med Chem 5:1609-1622.

Ghosh AK, Gemma S, Tang J (2008) beta-Secretase as a therapeutic target for Alzheimer's disease. Neurotherapeutics 5:399-408.
Ghosh AK, Brindisi M, Tang J (2012) Developing beta-secretase inhibitors for treatment of Alzheimer's disease. Journal of neurochemistry 120 Suppl 1:71-83.
Glass DA, 2nd, Karsenty G (2006) Canonical Wnt signaling in osteoblasts is required for osteoclast differentiation. Annals of the New York Academy of Sciences 1068:117-130.
Goldgaber D, Harris HW, Hla T, Maciag T, Donnelly RJ, Jacobsen JS, Vitek MP, Gajdusek DC (1989) Interleukin 1 regulates synthesis of amyloid beta-protein precursor mRNA in human endothelial cells. Proceedings of the National Academy of Sciences of the United States of America 86:7606-7610.
Gray CW, Patel AJ (1993) Regulation of beta-amyloid precursor protein isoform mRNAs by transforming growth factor-beta 1 and interleukin-1 beta in astrocytes. Brain research Molecular brain research 19:251-256.
Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995a) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. The Journal of biological chemistry 270:13757-13765.
Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G (1995b) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem 270:13757-13765.
Grunberg J, Walter J, Loetscher H, Deuschle U, Jacobsen H, Haass C (1998) Alzheimer's disease associated presenilin-1 holoprotein and its 18-20 kDa C-terminal fragment are death substrates for proteases of the caspase family. Biochemistry 37:2263-2270.
Guareschi S, Cova E, Cereda C, Ceroni M, Donetti E, Bosco DA, Trotti D, Pasinelli P (2012) An over-oxidized form of superoxide dismutase found in sporadic amyotrophic lateral sclerosis with bulbar onset shares a toxic mechanism with mutant SOD1. Proceedings of the National Academy of Sciences of the United States of America 109:5074-5079.
Gulen MF, Kang Z, Bulek K, Youzhong W, Kim TW, Chen Y, Altuntas CZ, Sass Bak-Jensen K, McGeachy MJ, Do JS, Xiao H, Delgoffe GM, Min B, Powell JD, Tuohy VK, Cua DJ, Li X (2010) The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. Immunity 32:54-66.
Guma M, Ronacher L, Liu-Bryan R, Takai S, Karin M, Corr M (2009) Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. Arthritis Rheum 60:3642-3650.
Gupta-Rossi N, Six E, LeBail O, Logeat F, Chastagner P, Olry A, Israel A, Brou C (2004a) Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J Cell Biol 166:73-83.
Gupta-Rossi N, Six E, LeBail O, Logeat F, Chastagner P, Olry A, Israel A, Brou C (2004b) Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. The Journal of cell biology 166:73-83.
Haapasalo A, Kovacs DM (2011) The many substrates of presenilin/gamma-secretase. Journal of Alzheimer's disease : JAD 25:3-28.
Haapasalo A, Kim DY, Carey BW, Turunen MK, Pettingell WH, Kovacs DM (2007) Presenilin/gamma-secretase-mediated cleavage regulates association of leukocyte-common antigen-related (LAR) receptor tyrosine phosphatase with beta-catenin. The Journal of biological chemistry 282:9063-9072.
Haass C, Selkoe DJ (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell 75:1039-1042.
Hansson CA, Popescu BO, Laudon H, Cedazo-Minguez A, Popescu LM, Winblad B, Ankarcrona M (2006) Caspase cleaved presenilin-1 is part of active gamma-secretase complexes. J Neurochem 97:356-364.

Harrison SM, Harper AJ, Hawkins J, Duddy G, Grau E, Pugh PL, Winter PH, Shilliam CS, Hughes ZA, Dawson LA, Gonzalez MI, Upton N, Pangalos MN, Dingwall C (2003) BACE1 (beta-secretase) transgenic and knockout mice: identification of neurochemical deficits and behavioral changes. Mol Cell Neurosci 24:646-655.

Hass MR, Sato C, Kopan R, Zhao G (2008) Presenilin: RIP and beyond. Semin Cell Dev Biol.

He G, Luo W, Li P, Remmers C, Netzer WJ, Hendrick J, Bettayeb K, Flajolet M, Gorelick F, Wennogle LP, Greengard P (2010) Gamma-secretase activating protein is a therapeutic target for Alzheimer's disease. Nature 467:95-98.

Hebert SS, Serneels L, Tolia A, Craessaerts K, Derks C, Filippov MA, Muller U, De Strooper B (2006) Regulated intramembrane proteolysis of amyloid precursor protein and regulation of expression of putative target genes. EMBO Rep 7:739-745.

Hebert SS, Serneels L, Dejaegere T, Horre K, Dabrowski M, Baert V, Annaert W, Hartmann D, De Strooper B (2004) Coordinated and widespread expression of gamma-secretase in vivo: evidence for size and molecular heterogeneity. Neurobiol Dis 17:260-272.

Hedskog L, Petersen CA, Svensson AI, Welander H, Tjernberg LO, Karlstrom H, Ankarcrona M (2011) gamma-Secretase complexes containing caspase-cleaved presenilin-1 increase intracellular Abeta(42) /Abeta(40) ratio. Journal of cellular and molecular medicine 15:2150-2163.

Hellberg K, Grimsrud PA, Kruse AC, Banaszak LJ, Ohlendorf DH, Bernlohr DA (2010) X-ray crystallographic analysis of adipocyte fatty acid binding protein (aP2) modified with 4-hydroxy-2-nonenal. Protein Sci 19:1480-1489.

Herreman A, Serneels L, Annaert W, Collen D, Schoonjans L, De Strooper B (2000) Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells. Nat Cell Biol 2:461-462.

Hicke L (2001) Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol 2:195-201.

Hitt BD, Jaramillo TC, Chetkovich DM, Vassar R (2010) BACE1-/- mice exhibit seizure activity that does not correlate with sodium channel level or axonal localization. Molecular neurodegeneration 5:31.

Ho A, Shen J (2011) Presenilins in synaptic function and disease. Trends Mol Med 17:617-624.

Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat Genet 29:301-305.

Honarnejad K, Herms J (2012) Presenilins: role in calcium homeostasis. The international journal of biochemistry & cell biology 44:1983-1986.

Hong L, Turner RT, 3rd, Koelsch G, Shin D, Ghosh AK, Tang J (2002) Crystal structure of memapsin 2 (beta-secretase) in complex with an inhibitor OM00-3. Biochemistry 41:10963-10967.

Hong L, Koelsch G, Lin X, Wu S, Terzyan S, Ghosh AK, Zhang XC, Tang J (2000) Structure of the protease domain of memapsin 2 (beta-secretase) complexed with inhibitor. Science 290:150-153.

Hsu H, Shu HB, Pan MG, Goeddel DV (1996a) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299-308.

Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV (1996b) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 4:387-396.

Huang H, He X (2008) Wnt/beta-catenin signaling: new (and old) players and new insights. Curr Opin Cell Biol 20:119-125.

Huang J, Gao X, Li S, Cao Z (1997a) Recruitment of IRAK to the interleukin 1 receptor complex requires interleukin 1 receptor accessory protein. Proc Natl Acad Sci U S A 94:12829-12832.

Huang J, Gao X, Li S, Cao Z (1997b) Recruitment of IRAK to the interleukin 1 receptor complex requires interleukin 1 receptor accessory protein. Proceedings of the National Academy of Sciences of the United States of America 94:12829-12832.

Hull C, McLean G, Wong F, Duriez PJ, Karsan A (2002) Lipopolysaccharide signals an endothelial apoptosis pathway through TNF receptor-associated factor 6-mediated activation of c-Jun NH2-terminal kinase. J Immunol 169:2611-2618.

Hutton M, Hardy J (1997) The presenilins and Alzheimer's disease. Hum Mol Genet 6:1639-1646.

Ikeda F, Dikic I (2008) Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep 9:536-542.

Inoue E, Deguchi-Tawarada M, Togawa A, Matsui C, Arita K, Katahira-Tayama S, Sato T, Yamauchi E, Oda Y, Takai Y (2009) Synaptic activity prompts gamma-secretase-mediated cleavage of EphA4 and dendritic spine formation. The Journal of cell biology 185:551-564.

Iqbal K, Liu F, Gong CX, Alonso Adel C, Grundke-Iqbal I (2009) Mechanisms of tau-induced neurodegeneration. Acta neuropathologica 118:53-69.

Jacobsen H, Reinhardt D, Brockhaus M, Bur D, Kocyba C, Kurt H, Grim MG, Baumeister R, Loetscher H (1999) The influence of endoproteolytic processing of familial Alzheimer's disease presenilin 2 on abeta42 amyloid peptide formation. J Biol Chem 274:35233-35239.

Jakob-Roetne R, Jacobsen H (2009) Alzheimer's disease: from pathology to therapeutic approaches. Angew Chem Int Ed Engl 48:3030-3059.

Jiang Z, Ninomiya-Tsuji J, Qian Y, Matsumoto K, Li X (2002) Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Molecular and cellular biology 22:7158-7167.

Jin H, Sanjo N, Uchihara T, Watabe K, St George-Hyslop P, Fraser PE, Mizusawa H (2010) Presenilin-1 holoprotein is an interacting partner of sarco endoplasmic reticulum calcium-ATPase and confers resistance to endoplasmic reticulum stress. Journal of Alzheimer's disease : JAD 20:261-273.

Johnston JA, Ward CL, Kopito RR (1998) Aggresomes: a cellular response to misfolded proteins. The Journal of cell biology 143:1883-1898.

Joosten LA (2010) Excessive interleukin-1 signaling determines the development of Th1 and Th17 responses in chronic inflammation. Arthritis Rheum 62:320-322.

Joosten LA, Netea MG, Fantuzzi G, Koenders MI, Helsen MM, Sparrer H, Pham CT, van der Meer JW, Dinarello CA, van den Berg WB (2009) Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. Arthritis Rheum 60:3651-3662.

Jung KM, Tan S, Landman N, Petrova K, Murray S, Lewis R, Kim PK, Kim DS, Ryu SH, Chao MV, Kim TW (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J Biol Chem 278:42161-42169.

Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. Nature immunology 9:361-368.

Kajiya H (2012) Calcium signaling in osteoclast differentiation and bone resorption. Adv Exp Med Biol 740:917-932.

Kametani F, Usami M, Tanaka K, Kume H, Mori H (2004) Mutant presenilin (A260V) affects Rab8 in PC12D cell. Neurochemistry international 44:313-320.

Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell 15:535-548.

Kang DE, Soriano S, Xia X, Eberhart CG, De Strooper B, Zheng H, Koo EH (2002) Presenilin couples the paired phosphorylation of beta-catenin independent of axin: implications for beta-catenin activation in tumorigenesis. Cell 110:751-762.

Kang DE, Soriano S, Frosch MP, Collins T, Naruse S, Sisodia SS, Leibowitz G, Levine F, Koo EH (1999) Presenilin 1 facilitates the constitutive turnover of beta-catenin: differential activity of Alzheimer's disease-linked PS1 mutants in the beta-catenin-signaling pathway. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:4229-4237.

Kang RS, Daniels CM, Francis SA, Shih SC, Salerno WJ, Hicke L, Radhakrishnan I (2003) Solution structure of a CUE-ubiquitin complex reveals a conserved mode of ubiquitin binding. Cell 113:621-630.

Kang TH, Guibinga GH, Jinnah HA, Friedmann T (2011) HPRT deficiency coordinately dysregulates canonical Wnt and presenilin-1 signaling: a neuro-developmental regulatory role for a housekeeping gene? PLoS ONE 6:e16572.

Kawai T, Akira S (2006) TLR signaling. Cell death and differentiation 13:816-825.

Khandelwal A, Chandu D, Roe CM, Kopan R, Quatrano RS (2007) Moonlighting activity of presenilin in plants is independent of gamma-secretase and evolutionarily conserved. Proceedings of the National Academy of Sciences of the United States of America 104:13337-13342.

Killick R, Pollard CC, Asuni AA, Mudher AK, Richardson JC, Rupniak HT, Sheppard PW, Varndell IM, Brion JP, Levey AI, Levy OA, Vestling M, Cowburn R, Lovestone S, Anderton BH (2001) Presenilin 1 independently regulates beta-catenin stability and transcriptional activity. The Journal of biological chemistry 276:48554-48561.

Kim TW, Pettingell WH, Jung YK, Kovacs DM, Tanzi RE (1997a) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science 277:373-376.

Kim TW, Pettingell WH, Hallmark OG, Moir RD, Wasco W, Tanzi RE (1997b) Endoproteolytic cleavage and proteasomal degradation of presenilin 2 in transfected cells. The Journal of biological chemistry 272:11006-11010.

Kimberly WT, Zheng JB, Town T, Flavell RA, Selkoe DJ (2005) Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5533-5543.

Kimberly WT, LaVoie MJ, Ostaszewski BL, Ye W, Wolfe MS, Selkoe DJ (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. Proc Natl Acad Sci U S A 100:6382-6387.

Kinoshita A, Uemura K, Ando K (2006) [Transcriptional regulation of synaptic proteins by presenilin/gamma-secretase]. Seikagaku 78:965-972.

Kirschenbaum F, Hsu SC, Cordell B, McCarthy JV (2001a) Glycogen synthase kinase-3beta regulates presenilin 1 C-terminal fragment levels. J Biol Chem 276:30701-30707.

Kirschenbaum F, Hsu SC, Cordell B, McCarthy JV (2001b) Substitution of a glycogen synthase kinase-3beta phosphorylation site in presenilin 1 separates presenilin function from beta-catenin signaling. J Biol Chem 276:7366-7375.

Kishida S, Sanjo H, Akira S, Matsumoto K, Ninomiya-Tsuji J (2005) TAK1-binding protein 2 facilitates ubiquitination of TRAF6 and assembly of TRAF6 with IKK in the IL-1 signaling pathway. Genes to cells : devoted to molecular & cellular mechanisms 10:447-454.

Kobayashi T, Kim TS, Jacob A, Walsh MC, Kadono Y, Fuentes-Panana E, Yoshioka T, Yoshimura A, Yamamoto M, Kaisho T, Akira S, Monroe JG, Choi Y (2009) TRAF6 is required for generation of the B-1a B cell compartment as well as T cell-dependent and -independent humoral immune responses. PLoS ONE 4:e4736.

Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, Wesche H, Martin MU (2004) Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. The Journal of biological chemistry 279:5227-5236.

Kopan R, Ilagan MX (2004) Gamma-secretase: proteasome of the membrane? Nat Rev Mol Cell Biol 5:499-504.
Korchnak AC, Zhan Y, Aguilar MT, Chadee DN (2009) Cytokine-induced activation of mixed lineage kinase 3 requires TRAF2 and TRAF6. Cellular signalling 21:1620-1625.
Kovacs I, Lentini KM, Ingano LM, Kovacs DM (2006) Presenilin 1 forms aggresomal deposits in response to heat shock. Journal of molecular neuroscience : MN 29:9-19.
Kumar S, Walter J (2011) Phosphorylation of amyloid beta (Abeta) peptides - a trigger for formation of toxic aggregates in Alzheimer's disease. Aging (Albany NY) 3:803-812.
Kuo LH, Hu MK, Hsu WM, Tung YT, Wang BJ, Tsai WW, Yen CT, Liao YF (2008) Tumor necrosis factor-alpha-elicited stimulation of gamma-secretase is mediated by c-Jun N-terminal kinase-dependent phosphorylation of presenilin and nicastrin. Mol Biol Cell 19:4201-4212.
Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ (2000) Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. The American journal of pathology 157:435-448.
Lacey DL et al. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165-176.
Lai MT, Chen E, Crouthamel MC, DiMuzio-Mower J, Xu M, Huang Q, Price E, Register RB, Shi XP, Donoviel DB, Bernstein A, Hazuda D, Gardell SJ, Li YM (2003) Presenilin-1 and presenilin-2 exhibit distinct yet overlapping gamma-secretase activities. J Biol Chem 278:22475-22481.
Lambert JC, Mann DM, Harris JM, Chartier-Harlin MC, Cumming A, Coates J, Lemmon H, StClair D, Iwatsubo T, Lendon C (2001) The -48 C/T polymorphism in the presenilin 1 promoter is associated with an increased risk of developing Alzheimer's disease and an increased Abeta load in brain. J Med Genet 38:353-355.
Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG (2007a) Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem 282:4102-4112.
Lamothe B, Webster WK, Gopinathan A, Besse A, Campos AD, Darnay BG (2007b) TRAF6 ubiquitin ligase is essential for RANKL signaling and osteoclast differentiation. Biochemical and biophysical research communications 359:1044-1049.
Lamothe B, Campos AD, Webster WK, Gopinathan A, Hur L, Darnay BG (2008) The RING domain and first zinc finger of TRAF6 coordinate signaling by interleukin-1, lipopolysaccharide, and RANKL. J Biol Chem 283:24871-24880.
Lau KF, Howlett DR, Kesavapany S, Standen CL, Dingwall C, McLoughlin DM, Miller CC (2002) Cyclin-dependent kinase-5/p35 phosphorylates Presenilin 1 to regulate carboxy-terminal fragment stability. Mol Cell Neurosci 20:13-20.
Lauderback CM, Hackett JM, Huang FF, Keller JN, Szweda LI, Markesbery WR, Butterfield DA (2001) The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: the role of Abeta1-42. Journal of neurochemistry 78:413-416.
Lazarov O, Morfini GA, Pigino G, Gadadhar A, Chen X, Robinson J, Ho H, Brady ST, Sisodia SS (2007) Impairments in fast axonal transport and motor neuron deficits in transgenic mice expressing familial Alzheimer's disease-linked mutant presenilin 1. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:7011-7020.
Lee JH, Yu WH, Kumar A, Lee S, Mohan PS, Peterhoff CM, Wolfe DM, Martinez-Vicente M, Massey AC, Sovak G, Uchiyama Y, Westaway D, Cuervo AM, Nixon RA (2010) Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. Cell 141:1146-1158.

Lee JT, Wheeler TC, Li L, Chin LS (2008) Ubiquitination of alpha-synuclein by Siah-1 promotes alpha-synuclein aggregation and apoptotic cell death. Human molecular genetics 17:906-917.
Lee NK, Lee SY (2002) Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs). J Biochem Mol Biol 35:61-66.
Lee S, Das HK (2008) Inhibition of basal activity of c-jun-NH2-terminal kinase (JNK) represses the expression of presenilin-1 by a p53-dependent mechanism. Brain research 1207:19-31.
Lee TH, Shank J, Cusson N, Kelliher MA (2004) The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. The Journal of biological chemistry 279:33185-33191.
Legler DF, Micheau O, Doucey MA, Tschopp J, Bron C (2003) Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. Immunity 18:655-664.
Li J, Pauley AM, Myers RL, Shuang R, Brashler JR, Yan R, Buhl AE, Ruble C, Gurney ME (2002) SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. J Neurochem 82:1540-1548.
Li Q, Harraz MM, Zhou W, Zhang LN, Ding W, Zhang Y, Eggleston T, Yeaman C, Banfi B, Engelhardt JF (2006a) Nox2 and Rac1 regulate H2O2-dependent recruitment of TRAF6 to endosomal interleukin-1 receptor complexes. Molecular and cellular biology 26:140-154.
Li Q, Harraz MM, Zhou W, Zhang LN, Ding W, Zhang Y, Eggleston T, Yeaman C, Banfi B, Engelhardt JF (2006b) Nox2 and Rac1 regulate H2O2-dependent recruitment of TRAF6 to endosomal interleukin-1 receptor complexes. Mol Cell Biol 26:140-154.
Li W, West N, Colla E, Pletnikova O, Troncoso JC, Marsh L, Dawson TM, Jakala P, Hartmann T, Price DL, Lee MK (2005) Aggregation promoting C-terminal truncation of alpha-synuclein is a normal cellular process and is enhanced by the familial Parkinson's disease-linked mutations. Proceedings of the National Academy of Sciences of the United States of America 102:2162-2167.
Li YM, Lai MT, Xu M, Huang Q, DiMuzio-Mower J, Sardana MK, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000a) Presenilin 1 is linked with gamma-secretase activity in the detergent solubilized state. Proc Natl Acad Sci U S A 97:6138-6143.
Li YM, Xu M, Lai MT, Huang Q, Castro JL, DiMuzio-Mower J, Harrison T, Lellis C, Nadin A, Neduvelil JG, Register RB, Sardana MK, Shearman MS, Smith AL, Shi XP, Yin KC, Shafer JA, Gardell SJ (2000b) Photoactivated gamma-secretase inhibitors directed to the active site covalently label presenilin 1. Nature 405:689-694.
Liao YF, Wang BJ, Cheng HT, Kuo LH, Wolfe MS (2004) Tumor necrosis factor-alpha, interleukin-1beta, and interferon-gamma stimulate gamma-secretase-mediated cleavage of amyloid precursor protein through a JNK-dependent MAPK pathway. The Journal of biological chemistry 279:49523-49532.
Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:5709-5714.
Limon-Mortes MC, Mora-Santos M, Espina A, Pintor-Toro JA, Lopez-Roman A, Tortolero M, Romero F (2008) UV-induced degradation of securin is mediated by SKP1-CUL1-beta TrCP E3 ubiquitin ligase. Journal of cell science 121:1825-1831.
Lleo A (2008) Activity of gamma-secretase on substrates other than APP. Curr Top Med Chem 8:9-16.
Lleo A, Saura CA (2011) gamma-secretase substrates and their implications for drug development in Alzheimer's disease. Curr Top Med Chem 11:1513-1527.

Loetscher H, Deuschle U, Brockhaus M, Reinhardt D, Nelboeck P, Mous J, Grunberg J, Haass C, Jacobsen H (1997) Presenilins are processed by caspase-type proteases. J Biol Chem 272:20655-20659.
Lomaga MA et al. (1999a) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes & development 13:1015-1024.
Lomaga MA et al. (1999b) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 13:1015-1024.
Loniewski KJ, Patial S, Parameswaran N (2007) Sensitivity of TLR4- and -7-induced NF kappa B1 p105-TPL2-ERK pathway to TNF-receptor-associated-factor-6 revealed by RNAi in mouse macrophages. Mol Immunol 44:3715-3723.
Loughran G, Healy NC, Kiely PA, Huigsloot M, Kedersha NL, O'Connor R (2005) Mystique is a new insulin-like growth factor-I-regulated PDZ-LIM domain protein that promotes cell attachment and migration and suppresses Anchorage-independent growth. Molecular biology of the cell 16:1811-1822.
Louvi A, Artavanis-Tsakonas S (2006) Notch signalling in vertebrate neural development. Nat Rev Neurosci 7:93-102.
Lovell MA, Xie C, Markesbery WR (2001) Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. Neurobiology of aging 22:187-194.
Luo Y, Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R (2001) Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. Nat Neurosci 4:231-232.
Ma G, Li T, Price DL, Wong PC (2005) APH-1a is the principal mammalian APH-1 isoform present in gamma-secretase complexes during embryonic development. J Neurosci 25:192-198.
Madura K (2002) The ubiquitin-associated (UBA) domain: on the path from prudence to prurience. Cell Cycle 1:235-244.
Maesako M, Uemura K, Kuzuya A, Sasaki K, Asada M, Watanabe K, Ando K, Kubota M, Akiyama H, Takahashi R, Kihara T, Shimohama S, Kinoshita A (2012) Gain of function by phosphorylation in Presenilin 1-mediated regulation of insulin signaling. Journal of neurochemistry 121:964-973.
Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O (2009) Nuclear signalling by tumour-associated antigen EpCAM. Nat Cell Biol 11:162-171.
Mah AL, Perry G, Smith MA, Monteiro MJ (2000) Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation. The Journal of cell biology 151:847-862.
Mancilla J, Ikejima T, Dinarello CA (1992) Glycosylation of the interleukin-1 receptor type I is required for optimal binding of interleukin-1. Lymphokine Cytokine Res 11:197-205.
Marambaud P, Ancolio K, Lopez-Perez E, Checler F (1998) Proteasome inhibitors prevent the degradation of familial Alzheimer's disease-linked presenilin 1 and potentiate A beta 42 recovery from human cells. Molecular medicine 4:147-157.
Martin JB (1999) Molecular basis of the neurodegenerative disorders. N Engl J Med 340:1970-1980.
Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27:229-265.
Marutani T, Maeda T, Tanabe C, Zou K, Araki W, Kokame K, Michikawa M, Komano H (2011) ER-stress-inducible Herp, facilitates the degradation of immature nicastrin. Biochimica et biophysica acta 1810:790-798.
Massey LK, Mah AL, Monteiro MJ (2005) Ubiquilin regulates presenilin endoproteolysis and modulates gamma-secretase components, Pen-2 and nicastrin. Biochem J 391:513-525.

Massey LK, Mah AL, Ford DL, Miller J, Liang J, Doong H, Monteiro MJ (2004) Overexpression of ubiquilin decreases ubiquitination and degradation of presenilin proteins. Journal of Alzheimer's disease : JAD 6:79-92.

Mastrangelo P, Mathews PM, Chishti MA, Schmidt SD, Gu Y, Yang J, Mazzella MJ, Coomaraswamy J, Horne P, Strome B, Pelly H, Levesque G, Ebeling C, Jiang Y, Nixon RA, Rozmahel R, Fraser PE, St George-Hyslop P, Carlson GA, Westaway D (2005) Dissociated phenotypes in presenilin transgenic mice define functionally distinct gamma-secretases. Proceedings of the National Academy of Sciences of the United States of America 102:8972-8977.

Matsuki T, Nakae S, Sudo K, Horai R, Iwakura Y (2006) Abnormal T cell activation caused by the imbalance of the IL-1/IL-1R antagonist system is responsible for the development of experimental autoimmune encephalomyelitis. International immunology 18:399-407.

May P, Reddy YK, Herz J (2002) Proteolytic processing of low density lipoprotein receptor-related protein mediates regulated release of its intracellular domain. J Biol Chem 277:18736-18743.

Mbefo MK, Paleologou KE, Boucharaba A, Oueslati A, Schell H, Fournier M, Olschewski D, Yin G, Zweckstetter M, Masliah E, Kahle PJ, Hirling H, Lashuel HA (2010) Phosphorylation of synucleins by members of the Polo-like kinase family. The Journal of biological chemistry 285:2807-2822.

McCarthy JV, Twomey C, Wujek P (2009a) Presenilin-dependent regulated intramembrane proteolysis and gamma-secretase activity. Cellular and molecular life sciences : CMLS 66:1534-1555.

McCarthy JV, Twomey C, Wujek P (2009b) Presenilin-dependent regulated intramembrane proteolysis and gamma-secretase activity. Cell Mol Life Sci.

McGrath LT, McGleenon BM, Brennan S, McColl D, Mc IS, Passmore AP (2001) Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde. Qjm 94:485-490.

Mentaverri R, Kamel S, Brazier M (2003) Involvement of capacitive calcium entry and calcium store refilling in osteoclastic survival and bone resorption process. Cell Calcium 34:169-175.

Micheau O, Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell 114:181-190.

Mitsuda N, Ohkubo N, Tamatani M, Lee YD, Taniguchi M, Namikawa K, Kiyama H, Yamaguchi A, Sato N, Sakata K, Ogihara T, Vitek MP, Tohyama M (2001) Activated cAMP-response element-binding protein regulates neuronal expression of presenilin-1. The Journal of biological chemistry 276:9688-9698.

Moehlmann T, Winkler E, Xia X, Edbauer D, Murrell J, Capell A, Kaether C, Zheng H, Ghetti B, Haass C, Steiner H (2002) Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Abeta 42 production. Proceedings of the National Academy of Sciences of the United States of America 99:8025-8030.

Morfini G, Szebenyi G, Elluru R, Ratner N, Brady ST (2002) Glycogen synthase kinase 3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. The EMBO journal 21:281-293.

Motegi H, Shimo Y, Akiyama T, Inoue J (2011) TRAF6 negatively regulates the Jak1-Erk pathway in interleukin-2 signaling. Genes to cells : devoted to molecular & cellular mechanisms 16:179-189.

Murakami D, Okamoto I, Nagano O, Kawano Y, Tomita T, Iwatsubo T, De Strooper B, Yumoto E, Saya H (2003) Presenilin-dependent gamma-secretase activity mediates the intramembranous cleavage of CD44. Oncogene 22:1511-1516.

Murray IV, Giasson BI, Quinn SM, Koppaka V, Axelsen PH, Ischiropoulos H, Trojanowski JQ, Lee VM (2003) Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. Biochemistry 42:8530-8540.

Naito A, Azuma S, Tanaka S, Miyazaki T, Takaki S, Takatsu K, Nakao K, Nakamura K, Katsuki M, Yamamoto T, Inoue J (1999) Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes Cells 4:353-362.

Nakayama K, Ohkawara T, Hiratochi M, Koh CS, Nagase H (2008) The intracellular domain of amyloid precursor protein induces neuron-specific apoptosis. Neuroscience letters 444:127-131.

Neely KM, Green KN, LaFerla FM (2011) Presenilin is necessary for efficient proteolysis through the autophagy-lysosome system in a gamma-secretase-independent manner. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:2781-2791.

Nelson O, Supnet C, Liu H, Bezprozvanny I (2010) Familial Alzheimer's disease mutations in presenilins: effects on endoplasmic reticulum calcium homeostasis and correlation with clinical phenotypes. Journal of Alzheimer's disease : JAD 21:781-793.

Nelson O, Tu H, Lei T, Bentahir M, de Strooper B, Bezprozvanny I (2007) Familial Alzheimer disease-linked mutations specifically disrupt Ca2+ leak function of presenilin 1. The Journal of clinical investigation 117:1230-1239.

Nelson O, Supnet C, Tolia A, Horre K, De Strooper B, Bezprozvanny I (2011) Mutagenesis mapping of the presenilin 1 calcium leak conductance pore. The Journal of biological chemistry 286:22339-22347.

Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, Funk CJ, Mason RJ, Kullberg BJ, Rubartelli A, van der Meer JW, Dinarello CA (2009) Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. Blood 113:2324-2335.

Newman M, Nornes S, Martins RN, Lardelli MT (2012) Robust homeostasis of Presenilin1 protein levels by transcript regulation. Neuroscience letters 519:14-19.

Nguyen LT, Duncan GS, Mirtsos C, Ng M, Speiser DE, Shahinian A, Marino MW, Mak TW, Ohashi PS, Yeh WC (1999) TRAF2 deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. Immunity 11:379-389.

Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R (2005) A genomic and functional inventory of deubiquitinating enzymes. Cell 123:773-786.

Noll E, Medina M, Hartley D, Zhou J, Perrimon N, Kosik KS (2000) Presenilin affects arm/beta-catenin localization and function in Drosophila. Developmental biology 227:450-464.

Nornes S, Newman M, Verdile G, Wells S, Stoick-Cooper CL, Tucker B, Frederich-Sleptsova I, Martins R, Lardelli M (2008) Interference with splicing of Presenilin transcripts has potent dominant negative effects on Presenilin activity. Human molecular genetics 17:402-412.

Nunan J, Small DH (2000) Regulation of APP cleavage by alpha-, beta- and gamma-secretases. FEBS Lett 483:6-10.

Nyabi O, Bentahir M, Horre K, Herreman A, Gottardi-Littell N, Van Broeckhoven C, Merchiers P, Spittaels K, Annaert W, De Strooper B (2003) Presenilins mutated at Asp-257 or Asp-385 restore Pen-2 expression and Nicastrin glycosylation but remain catalytically inactive in the absence of wild type Presenilin. The Journal of biological chemistry 278:43430-43436.

O'Neill LA, Bowie AG (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7:353-364.

Oda A, Tamaoka A, Araki W (2010) Oxidative stress up-regulates presenilin 1 in lipid rafts in neuronal cells. Journal of neuroscience research 88:1137-1145.

Oeckinghaus A, Wegener E, Welteke V, Ferch U, Arslan SC, Ruland J, Scheidereit C, Krappmann D (2007) Malt1 ubiquitination triggers NF-kappaB signaling upon T-cell activation. Embo J 26:4634-4645.

Ohkawara T, Nagase H, Koh CS, Nakayama K (2011) The amyloid precursor protein intracellular domain alters gene expression and induces neuron-specific apoptosis. Gene 475:1-9.

Ohno M, Sametsky EA, Younkin LH, Oakley H, Younkin SG, Citron M, Vassar R, Disterhoft JF (2004) BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. Neuron 41:27-33.

Ohta K, Mizuno A, Li S, Itoh M, Ueda M, Ohta E, Hida Y, Wang MX, Furoi M, Tsuzuki Y, Sobajima M, Bohmoto Y, Fukushima T, Kobori M, Inuzuka T, Nakagawa T (2011) Endoplasmic reticulum stress enhances gamma-secretase activity. Biochemical and biophysical research communications 416:362-366.

Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, Dinarello CA, Fujita M (2010) Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. The Journal of biological chemistry 285:6477-6488.

Okochi M, Steiner H, Fukumori A, Tanii H, Tomita T, Tanaka T, Iwatsubo T, Kudo T, Takeda M, Haass C (2002) Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. The EMBO journal 21:5408-5416.

Ordureau A, Smith H, Windheim M, Peggie M, Carrick E, Morrice N, Cohen P (2008) The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. The Biochemical journal 409:43-52.

Oueslati A, Fournier M, Lashuel HA (2010) Role of post-translational modifications in modulating the structure, function and toxicity of alpha-synuclein: implications for Parkinson's disease pathogenesis and therapies. Prog Brain Res 183:115-145.

Ounallah-Saad H, Beeri R, Goshen I, Yirmiya R, Renbaum P, Levy-Lahad E (2009) Transcriptional regulation of the murine Presenilin-2 gene reveals similarities and differences to its human orthologue. Gene 446:81-89.

Ozaki T, Li Y, Kikuchi H, Tomita T, Iwatsubo T, Nakagawara A (2006) The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis. Biochemical and biophysical research communications 351:57-63.

Pardossi-Piquard R, Petit A, Kawarai T, Sunyach C, Alves da Costa C, Vincent B, Ring S, D'Adamio L, Shen J, Muller U, St George Hyslop P, Checler F (2005) Presenilin-dependent transcriptional control of the Abeta-degrading enzyme neprilysin by intracellular domains of betaAPP and APLP. Neuron 46:541-554.

Parisiadou L, Fassa A, Fotinopoulou A, Bethani I, Efthimiopoulos S (2004) Presenilin 1 and cadherins: stabilization of cell-cell adhesion and proteolysis-dependent regulation of transcription. Neuro-degenerative diseases 1:184-191.

Pastorcic M, Das HK (2002) Activation of transcription of the human presenilin 1 gene by 12-O-tetradecanoylphorbol 13-acetate. European journal of biochemistry / FEBS 269:5956-5962.

Pastorcic M, Das HK (2004) Alternative initiation of transcription of the human presenilin 1 gene in SH-SY5Y and SK-N-SH cells. The role of Ets factors in the regulation of presenilin 1. European journal of biochemistry / FEBS 271:4485-4494.

Pastorcic M, Das HK (2007a) Analysis of transcriptional modulation of the presenilin 1 gene promoter by ZNF237, a candidate binding partner of the Ets transcription factor ERM. Brain research 1128:21-32.

Pastorcic M, Das HK (2007b) The C-terminal region of CHD3/ZFH interacts with the CIDD region of the Ets transcription factor ERM and represses transcription of the human presenilin 1 gene. The FEBS journal 274:1434-1448.

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP (2003) A proteomics approach to understanding protein ubiquitination. Nat Biotechnol 21:921-926.
Perluigi M, Sultana R, Cenini G, Di Domenico F, Memo M, Pierce WM, Coccia R, Butterfield DA (2009) Redox proteomics identification of 4-hydroxynonenal-modified brain proteins in Alzheimer's disease: Role of lipid peroxidation in Alzheimer's disease pathogenesis. Proteomics Clin Appl 3:682-693.
Pickart CM (2001) Mechanisms underlying ubiquitination. Annu Rev Biochem 70:503-533.
Pickart CM (2004) Back to the future with ubiquitin. Cell 116:181-190.
Pigino G, Morfini G, Pelsman A, Mattson MP, Brady ST, Busciglio J (2003) Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:4499-4508.
Ponting CP (2000a) Proteins of the endoplasmic-reticulum-associated degradation pathway: domain detection and function prediction. Biochem J 351 Pt 2:527-535.
Ponting CP (2000b) Proteins of the endoplasmic-reticulum-associated degradation pathway: domain detection and function prediction. The Biochemical journal 351 Pt 2:527-535.
Powell JC, Twomey C, Jain R, McCarthy JV (2009) Association between Presenilin-1 and TRAF6 modulates regulated intramembrane proteolysis of the p75NTR neurotrophin receptor. J Neurochem 108:216-230.
Prag G, Misra S, Jones EA, Ghirlando R, Davies BA, Horazdovsky BF, Hurley JH (2003) Mechanism of ubiquitin recognition by the CUE domain of Vps9p. Cell 113:609-620.
Prager K, Wang-Eckhardt L, Fluhrer R, Killick R, Barth E, Hampel H, Haass C, Walter J (2007) A structural switch of presenilin 1 by glycogen synthase kinase 3beta-mediated phosphorylation regulates the interaction with beta-catenin and its nuclear signaling. J Biol Chem 282:14083-14093.
Qian Y, Commane M, Ninomiya-Tsuji J, Matsumoto K, Li X (2001) IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. The Journal of biological chemistry 276:41661-41667.
Rahighi S, Ikeda F, Kawasaki M, Akutsu M, Suzuki N, Kato R, Kensche T, Uejima T, Bloor S, Komander D, Randow F, Wakatsuki S, Dikic I (2009) Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. Cell 136:1098-1109.
Rajapaksha TW, Eimer WA, Bozza TC, Vassar R (2011) The Alzheimer's beta-secretase enzyme BACE1 is required for accurate axon guidance of olfactory sensory neurons and normal glomerulus formation in the olfactory bulb. Molecular neurodegeneration 6:88.
Ratovitski T, Slunt HH, Thinakaran G, Price DL, Sisodia SS, Borchelt DR (1997) Endoproteolytic processing and stabilization of wild-type and mutant presenilin. J Biol Chem 272:24536-24541.
Reed TT, Pierce WM, Markesbery WR, Butterfield DA (2009) Proteomic identification of HNE-bound proteins in early Alzheimer disease: Insights into the role of lipid peroxidation in the progression of AD. Brain research 1274:66-76.
Rogers JT, Leiter LM, McPhee J, Cahill CM, Zhan SS, Potter H, Nilsson LN (1999) Translation of the alzheimer amyloid precursor protein mRNA is up-regulated by interleukin-1 through 5'-untranslated region sequences. The Journal of biological chemistry 274:6421-6431.
Rosen DR (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 364:362.
Rott R, Szargel R, Haskin J, Shani V, Shainskaya A, Manov I, Liani E, Avraham E, Engelender S (2008) Monoubiquitylation of alpha-synuclein by seven in absentia homolog (SIAH) promotes its aggregation in dopaminergic cells. The Journal of biological chemistry 283:3316-3328.

Ryan KA, Pimplikar SW (2005) Activation of GSK-3 and phosphorylation of CRMP2 in transgenic mice expressing APP intracellular domain. The Journal of cell biology 171:327-335.

Ryu YS, Park SY, Jung MS, Yoon SH, Kwen MY, Lee SY, Choi SH, Radnaabazar C, Kim MK, Kim H, Kim K, Song WJ, Chung SH (2010) Dyrk1A-mediated phosphorylation of Presenilin 1: a functional link between Down syndrome and Alzheimer's disease. Journal of neurochemistry 115:574-584.

Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G (2006) Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Cell 127:185-197.

Sasai M, Tatematsu M, Oshiumi H, Funami K, Matsumoto M, Hatakeyama S, Seya T (2010) Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway. Mol Immunol 47:1283-1291.

Sato T, Diehl TS, Narayanan S, Funamoto S, Ihara Y, De Strooper B, Steiner H, Haass C, Wolfe MS (2007) Active gamma-secretase complexes contain only one of each component. J Biol Chem 282:33985-33993.

Saura CA, Tomita T, Soriano S, Takahashi M, Leem JY, Honda T, Koo EH, Iwatsubo T, Thinakaran G (2000) The nonconserved hydrophilic loop domain of presenilin (PS) is not required for PS endoproteolysis or enhanced abeta 42 production mediated by familial early onset Alzheimer's disease-linked PS variants. J Biol Chem 275:17136-17142.

Saura CA, Chen G, Malkani S, Choi SY, Takahashi RH, Zhang D, Gouras GK, Kirkwood A, Morris RG, Shen J (2005) Conditional inactivation of presenilin 1 prevents amyloid accumulation and temporarily rescues contextual and spatial working memory impairments in amyloid precursor protein transgenic mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:6755-6764.

Saura CA, Choi SY, Beglopoulos V, Malkani S, Zhang D, Shankaranarayana Rao BS, Chattarji S, Kelleher RJ, 3rd, Kandel ER, Duff K, Kirkwood A, Shen J (2004) Loss of presenilin function causes impairments of memory and synaptic plasticity followed by age-dependent neurodegeneration. Neuron 42:23-36.

Schauvliege R, Janssens S, Beyaert R (2007) Pellino proteins: novel players in TLR and IL-1R signalling. Journal of cellular and molecular medicine 11:453-461.

Scheper W, Zwart R, Baas F (2004) Rab6 membrane association is dependent of Presenilin 1 and cellular phosphorylation events. Brain research Molecular brain research 122:17-23.

Schroeter EH, Kisslinger JA, Kopan R (1998) Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 393:382-386.

Schroeter EH, Ilagan MX, Brunkan AL, Hecimovic S, Li YM, Xu M, Lewis HD, Saxena MT, De Strooper B, Coonrod A, Tomita T, Iwatsubo T, Moore CL, Goate A, Wolfe MS, Shearman M, Kopan R (2003) A presenilin dimer at the core of the gamma-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. Proceedings of the National Academy of Sciences of the United States of America 100:13075-13080.

Schultheiss U, Puschner S, Kremmer E, Mak TW, Engelmann H, Hammerschmidt W, Kieser A (2001) TRAF6 is a critical mediator of signal transduction by the viral oncogene latent membrane protein 1. The EMBO journal 20:5678-5691.

Sebban-Benin H, Pescatore A, Fusco F, Pascuale V, Gautheron J, Yamaoka S, Moncla A, Ursini MV, Courtois G (2007a) Identification of TRAF6-dependent NEMO polyubiquitination sites through analysis of a new NEMO mutation causing incontinentia pigmenti. Hum Mol Genet 16:2805-2815.

Sebban-Benin H, Pescatore A, Fusco F, Pascuale V, Gautheron J, Yamaoka S, Moncla A, Ursini MV, Courtois G (2007b) Identification of TRAF6-dependent NEMO polyubiquitination

sites through analysis of a new NEMO mutation causing incontinentia pigmenti. Human molecular genetics 16:2805-2815.
Serneels L, Dejaegere T, Craessaerts K, Horre K, Jorissen E, Tousseyn T, Hebert S, Coolen M, Martens G, Zwijsen A, Annaert W, Hartmann D, De Strooper B (2005) Differential contribution of the three Aph1 genes to gamma-secretase activity in vivo. Proc Natl Acad Sci U S A 102:1719-1724.
Serneels L et al. (2009) {gamma}-Secretase Heterogeneity in the Aph1 Subunit: Relevance for Alzheimer's Disease. Science.
Shen J, Bronson RT, Chen DF, Xia W, Selkoe DJ, Tonegawa S (1997) Skeletal and CNS defects in Presenilin-1-deficient mice. Cell 89:629-639.
Sheng JG, Mrak RE, Griffin WS (1997a) Glial-neuronal interactions in Alzheimer disease: progressive association of IL-1alpha+ microglia and S100beta+ astrocytes with neurofibrillary tangle stages. Journal of neuropathology and experimental neurology 56:285-290.
Sheng JG, Mrak RE, Griffin WS (1997b) Neuritic plaque evolution in Alzheimer's disease is accompanied by transition of activated microglia from primed to enlarged to phagocytic forms. Acta neuropathologica 94:1-5.
Shih Ie M, Wang TL (2007) Notch signaling, gamma-secretase inhibitors, and cancer therapy. Cancer Res 67:1879-1882.
Shih SC, Prag G, Francis SA, Sutanto MA, Hurley JH, Hicke L (2003a) A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. The EMBO journal 22:1273-1281.
Shih SC, Prag G, Francis SA, Sutanto MA, Hurley JH, Hicke L (2003b) A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. Embo J 22:1273-1281.
Shinohara M, Koga T, Okamoto K, Sakaguchi S, Arai K, Yasuda H, Takai T, Kodama T, Morio T, Geha RS, Kitamura D, Kurosaki T, Ellmeier W, Takayanagi H (2008) Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. Cell 132:794-806.
Shirotani K, Edbauer D, Prokop S, Haass C, Steiner H (2004) Identification of distinct gamma-secretase complexes with different APH-1 variants. J Biol Chem 279:41340-41345.
Shirotani K, Tomioka M, Kremmer E, Haass C, Steiner H (2007) Pathological activity of familial Alzheimer's disease-associated mutant presenilin can be executed by six different gamma-secretase complexes. Neurobiol Dis 27:102-107.
Siems WG, Hapner SJ, van Kuijk FJ (1996) 4-hydroxynonenal inhibits Na(+)-K(+)-ATPase. Free radical biology & medicine 20:215-223.
Sinha S et al. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402:537-540.
Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, Dower SK (2000) Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. The Journal of biological chemistry 275:4670-4678.
Slomnicki LP, Lesniak W (2008) A putative role of the Amyloid Precursor Protein Intracellular Domain (AICD) in transcription. Acta Neurobiol Exp (Wars) 68:219-228.
Smith SK, Anderson HA, Yu G, Robertson AG, Allen SJ, Tyler SJ, Naylor RL, Mason G, Wilcock GW, Roche PA, Fraser PE, Dawbarn D (2000) Identification of syntaxin 1A as a novel binding protein for presenilin-1. Brain research Molecular brain research 78:100-107.
Song W, Nadeau P, Yuan M, Yang X, Shen J, Yankner BA (1999) Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. Proceedings of the National Academy of Sciences of the United States of America 96:6959-6963.

Soriano S, Kang DE, Fu M, Pestell R, Chevallier N, Zheng H, Koo EH (2001) Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer factor-1 signaling independently of beta-amyloid precursor protein and notch processing. The Journal of cell biology 152:785-794.

Spasic D, Tolia A, Dillen K, Baert V, De Strooper B, Vrijens S, Annaert W (2006a) Presenilin-1 maintains a nine-transmembrane topology throughout the secretory pathway. The Journal of biological chemistry 281:26569-26577.

Spasic D, Tolia A, Dillen K, Baert V, De Strooper B, Vrijens S, Annaert W (2006b) Presenilin-1 maintains a nine-transmembrane topology throughout the secretory pathway. J Biol Chem 281:26569-26577.

Steiner H, Romig H, Grim MG, Philipp U, Pesold B, Citron M, Baumeister R, Haass C (1999a) The biological and pathological function of the presenilin-1 Deltaexon 9 mutation is independent of its defect to undergo proteolytic processing. J Biol Chem 274:7615-7618.

Steiner H, Duff K, Capell A, Romig H, Grim MG, Lincoln S, Hardy J, Yu X, Picciano M, Fechteler K, Citron M, Kopan R, Pesold B, Keck S, Baader M, Tomita T, Iwatsubo T, Baumeister R, Haass C (1999b) A loss of function mutation of presenilin-2 interferes with amyloid beta-peptide production and notch signaling. J Biol Chem 274:28669-28673.

Steiner H, Duff K, Capell A, Romig H, Grim MG, Lincoln S, Hardy J, Yu X, Picciano M, Fechteler K, Citron M, Kopan R, Pesold B, Keck S, Baader M, Tomita T, Iwatsubo T, Baumeister R, Haass C (1999c) A loss of function mutation of presenilin-2 interferes with amyloid beta-peptide production and notch signaling. The Journal of biological chemistry 274:28669-28673.

Subramaniam R, Roediger F, Jordan B, Mattson MP, Keller JN, Waeg G, Butterfield DA (1997) The lipid peroxidation product, 4-hydroxy-2-trans-nonenal, alters the conformation of cortical synaptosomal membrane proteins. Journal of neurochemistry 69:1161-1169.

Suga K, Tomiyama T, Mori H, Akagawa K (2004) Syntaxin 5 interacts with presenilin holoproteins, but not with their N- or C-terminal fragments, and affects beta-amyloid peptide production. The Biochemical journal 381:619-628.

Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol Cell 14:289-301.

Szargel R, Rott R, Eyal A, Haskin J, Shani V, Balan L, Wolosker H, Engelender S (2009) Synphilin-1A inhibits seven in absentia homolog (SIAH) and modulates alpha-synuclein monoubiquitylation and inclusion formation. The Journal of biological chemistry 284:11706-11716.

Tada K, Okazaki T, Sakon S, Kobarai T, Kurosawa K, Yamaoka S, Hashimoto H, Mak TW, Yagita H, Okumura K, Yeh WC, Nakano H (2001) Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. The Journal of biological chemistry 276:36530-36534.

Takahashi K, Niidome T, Akaike A, Kihara T, Sugimoto H (2008) Phosphorylation of amyloid precursor protein (APP) at Tyr687 regulates APP processing by alpha- and gamma-secretase. Biochem Biophys Res Commun 377:544-549.

Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T (2002) Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Developmental cell 3:889-901.

Takeo K, Watanabe N, Tomita T, Iwatsubo T (2012) Contribution of the gamma-secretase subunits to the formation of catalytic pore of presenilin 1 protein. The Journal of biological chemistry 287:25834-25843.

Tanaka T, Grusby MJ, Kaisho T (2007) PDLIM2-mediated termination of transcription factor NF-kappaB activation by intranuclear sequestration and degradation of the p65 subunit. Nature immunology 8:584-591.

Teranishi Y, Hur JY, Welander H, Franberg J, Aoki M, Winblad B, Frykman S, Tjernberg LO (2010) Affinity pulldown of gamma-secretase and associated proteins from human and rat brain. Journal of cellular and molecular medicine 14:2675-2686.

Theuns J, Remacle J, Killick R, Corsmit E, Vennekens K, Huylebroeck D, Cruts M, Van Broeckhoven C (2003) Alzheimer-associated C allele of the promoter polymorphism -22C>T causes a critical neuron-specific decrease of presenilin 1 expression. Human molecular genetics 12:869-877.

Theuns J, Del-Favero J, Dermaut B, van Duijn CM, Backhovens H, Van den Broeck MV, Serneels S, Corsmit E, Van Broeckhoven CV, Cruts M (2000) Genetic variability in the regulatory region of presenilin 1 associated with risk for Alzheimer's disease and variable expression. Human molecular genetics 9:325-331.

Thinakaran G, Harris CL, Ratovitski T, Davenport F, Slunt HH, Price DL, Borchelt DR, Sisodia SS (1997) Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. The Journal of biological chemistry 272:28415-28422.

Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron 17:181-190.

Tolia A, De Strooper B (2008) Structure and function of gamma-secretase. Semin Cell Dev Biol.

Tolia A, Chavez-Gutierrez L, De Strooper B (2006) Contribution of presenilin transmembrane domains 6 and 7 to a water-containing cavity in the gamma-secretase complex. J Biol Chem 281:27633-27642.

Tolia A, Horre K, De Strooper B (2008) Transmembrane Domain 9 of Presenilin Determines the Dynamic Conformation of the Catalytic Site of {gamma}-Secretase. J Biol Chem 283:19793-19803.

Tu H, Nelson O, Bezprozvanny A, Wang Z, Lee SF, Hao YH, Serneels L, De Strooper B, Yu G, Bezprozvanny I (2006) Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. Cell 126:981-993.

Twomey C, McCarthy JV (2006) Presenilin-1 is an unprimed glycogen synthase kinase-3beta substrate. FEBS Lett 580:4015-4020.

Twomey C, Qian S, McCarthy JV (2009) TRAF6 promotes ubiquitination and regulated intramembrane proteolysis of IL-1R1. Biochem Biophys Res Commun 381:418-423.

Uemura K, Kuzuya A, Shimozono Y, Aoyagi N, Ando K, Shimohama S, Kinoshita A (2007) GSK3beta activity modifies the localization and function of presenilin 1. J Biol Chem 282:15823-15832.

Underwood CK, Coulson EJ (2008) The p75 neurotrophin receptor. Int J Biochem Cell Biol 40:1664-1668.

Underwood CK, Reid K, May LM, Bartlett PF, Coulson EJ (2008) Palmitoylation of the C-terminal fragment of p75(NTR) regulates death signaling and is required for subsequent cleavage by gamma-secretase. Mol Cell Neurosci 37:346-358.

Vartanian T, Fischbach G, Miller R (1999) Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. Proceedings of the National Academy of Sciences of the United States of America 96:731-735.

Vassar R et al. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286:735-741.

Verstak B, Nagpal K, Bottomley SP, Golenbock DT, Hertzog PJ, Mansell A (2009) MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-

mediated NF-kappaB proinflammatory responses. The Journal of biological chemistry 284:24192-24203.
Vijay-Kumar S, Bugg CE, Cook WJ (1987) Structure of ubiquitin refined at 1.8 A resolution. J Mol Biol 194:531-544.
Viswanathan J, Haapasalo A, Bottcher C, Miettinen R, Kurkinen KM, Lu A, Thomas A, Maynard CJ, Romano D, Hyman BT, Berezovska O, Bertram L, Soininen H, Dantuma NP, Tanzi RE, Hiltunen M (2011) Alzheimer's disease-associated ubiquilin-1 regulates presenilin-1 accumulation and aggresome formation. Traffic 12:330-348.
Wakabayashi K, Tanji K, Mori F, Takahashi H (2007) The Lewy body in Parkinson's disease: molecules implicated in the formation and degradation of alpha-synuclein aggregates. Neuropathology : official journal of the Japanese Society of Neuropathology 27:494-506.
Wakabayashi T, Craessaerts K, Bammens L, Bentahir M, Borgions F, Herdewijn P, Staes A, Timmerman E, Vandekerckhove J, Rubinstein E, Boucheix C, Gevaert K, De Strooper B (2009) Analysis of the gamma-secretase interactome and validation of its association with tetraspanin-enriched microdomains. Nature cell biology 11:1340-1346.
Walsh MC, Kim GK, Maurizio PL, Molnar EE, Choi Y (2008) TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. PLoS One 3:e4064.
Walter J, Schindzielorz A, Grunberg J, Haass C (1999) Phosphorylation of presenilin-2 regulates its cleavage by caspases and retards progression of apoptosis. Proc Natl Acad Sci U S A 96:1391-1396.
Walter J, Capell A, Grunberg J, Pesold B, Schindzielorz A, Prior R, Podlisny MB, Fraser P, Hyslop PS, Selkoe DJ, Haass C (1996a) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. Mol Med 2:673-691.
Walter J, Capell A, Grunberg J, Pesold B, Schindzielorz A, Prior R, Podlisny MB, Fraser P, Hyslop PS, Selkoe DJ, Haass C (1996b) The Alzheimer's disease-associated presenilins are differentially phosphorylated proteins located predominantly within the endoplasmic reticulum. Molecular medicine 2:673-691.
Wang B, Yang W, Wen W, Sun J, Su B, Liu B, Ma D, Lv D, Wen Y, Qu T, Chen M, Sun M, Shen Y, Zhang X (2010) Gamma-secretase gene mutations in familial acne inversa. Science 330:1065.
Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346-351.
Wang L, Rahn JJ, Lun X, Sun B, Kelly JJ, Weiss S, Robbins SM, Forsyth PA, Senger DL (2008) Gamma-secretase represents a therapeutic target for the treatment of invasive glioma mediated by the p75 neurotrophin receptor. PLoS Biol 6:e289.
Wang R, Sweeney D, Gandy SE, Sisodia SS (1996) The profile of soluble amyloid beta protein in cultured cell media. Detection and quantification of amyloid beta protein and variants by immunoprecipitation-mass spectrometry. J Biol Chem 271:31894-31902.
Wang ZM, Lashuel HA (2012) Discovery of a Novel Aggregation Domain in the Huntingtin Protein: Implications for the Mechanisms of Htt Aggregation and Toxicity. Angew Chem Int Ed Engl.
Waschbusch D, Born S, Niediek V, Kirchgessner N, Tamboli IY, Walter J, Merkel R, Hoffmann B (2009) Presenilin 1 Affects Focal Adhesion Site Formation and Cell Force Generation via c-Src Transcriptional and Posttranslational Regulation. J Biol Chem 284:10138-10149.
Watanabe S, Nagano S, Duce J, Kiaei M, Li QX, Tucker SM, Tiwari A, Brown RH, Jr., Beal MF, Hayward LJ, Culotta VC, Yoshihara S, Sakoda S, Bush AI (2007) Increased affinity for

copper mediated by cysteine 111 in forms of mutant superoxide dismutase 1 linked to amyotrophic lateral sclerosis. Free radical biology & medicine 42:1534-1542.
Weidemann A, Eggert S, Reinhard FB, Vogel M, Paliga K, Baier G, Masters CL, Beyreuther K, Evin G (2002) A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. Biochemistry 41:2825-2835.
Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 7:837-847.
Willem M, Garratt AN, Novak B, Citron M, Kaufmann S, Rittger A, DeStrooper B, Saftig P, Birchmeier C, Haass C (2006) Control of peripheral nerve myelination by the beta-secretase BACE1. Science 314:664-666.
Wilson CA, Murphy DD, Giasson BI, Zhang B, Trojanowski JQ, Lee VM (2004) Degradative organelles containing mislocalized alpha-and beta-synuclein proliferate in presenilin-1 null neurons. The Journal of cell biology 165:335-346.
Wines-Samuelson M, Schulte EC, Smith MJ, Aoki C, Liu X, Kelleher RJ, 3rd, Shen J (2010) Characterization of age-dependent and progressive cortical neuronal degeneration in presenilin conditional mutant mice. PLoS ONE 5:e10195.
Winkler E, Hobson S, Fukumori A, Dumpelfeld B, Luebbers T, Baumann K, Haass C, Hopf C, Steiner H (2009) Purification, Pharmacological Modulation, and Biochemical Characterization of Interactors of Endogenous Human gamma-Secretase (dagger). Biochemistry.
Wolfe MS (2008a) gamma-Secretase in biology and medicine. Semin Cell Dev Biol.
Wolfe MS (2008b) Gamma-secretase: structure, function, and modulation for Alzheimer's disease. Curr Top Med Chem 8:2-8.
Wolfe MS (2009) gamma-Secretase in biology and medicine. Semin Cell Dev Biol 20:219-224.
Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Kimberly WT, Selkoe DJ (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398:513-517.
Wong BR, Besser D, Kim N, Arron JR, Vologodskaia M, Hanafusa H, Choi Y (1999) TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Molecular cell 4:1041-1049.
Wu G, Hubbard EJ, Kitajewski JK, Greenwald I (1998) Evidence for functional and physical association between Caenorhabditis elegans SEL-10, a Cdc4p-related protein, and SEL-12 presenilin. Proc Natl Acad Sci U S A 95:15787-15791.
Xia X, Qian S, Soriano S, Wu Y, Fletcher AM, Wang XJ, Koo EH, Wu X, Zheng H (2001) Loss of presenilin 1 is associated with enhanced beta-catenin signaling and skin tumorigenesis. Proceedings of the National Academy of Sciences of the United States of America 98:10863-10868.
Xiao F, Wang H, Fu X, Li Y, Wu Z (2012) TRAF6 promotes myogenic differentiation via the TAK1/p38 mitogen-activated protein kinase and Akt pathways. PLoS ONE 7:e34081.
Yamasaki A, Eimer S, Okochi M, Smialowska A, Kaether C, Baumeister R, Haass C, Steiner H (2006) The GxGD motif of presenilin contributes to catalytic function and substrate identification of gamma-secretase. J Neurosci 26:3821-3828.
Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrikson RL, Gurney ME (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402:533-537.
Yang J, Lin Y, Guo Z, Cheng J, Huang J, Deng L, Liao W, Chen Z, Liu Z, Su B (2001) The essential role of MEKK3 in TNF-induced NF-kappaB activation. Nature immunology 2:620-624.
Yang S, Li YP (2007) RGS10-null mutation impairs osteoclast differentiation resulting from the loss of [Ca2+]i oscillation regulation. Genes & development 21:1803-1816.

Yang WL, Wang J, Chan CH, Lee SW, Campos AD, Lamothe B, Hur L, Grabiner BC, Lin X, Darnay BG, Lin HK (2009) The E3 ligase TRAF6 regulates Akt ubiquitination and activation. Science 325:1134-1138.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proceedings of the National Academy of Sciences of the United States of America 95:3597-3602.

Ye H, Arron JR, Lamothe B, Cirilli M, Kobayashi T, Shevde NK, Segal D, Dzivenu OK, Vologodskaia M, Yim M, Du K, Singh S, Pike JW, Darnay BG, Choi Y, Wu H (2002) Distinct molecular mechanism for initiating TRAF6 signalling. Nature 418:443-447.

Yen ML, Hsu PN, Liao HJ, Lee BH, Tsai HF (2012) TRAF-6 dependent signaling pathway is essential for TNF-related apoptosis-inducing ligand (TRAIL) induces osteoclast differentiation. PLoS ONE 7:e38048.

Yonemura Y, Futai E, Yagishita S, Suo S, Tomita T, Iwatsubo T, Ishiura S (2011) Comparison of presenilin 1 and presenilin 2 gamma-secretase activities using a yeast reconstitution system. The Journal of biological chemistry 286:44569-44575.

Yu G, Chen F, Nishimura M, Steiner H, Tandon A, Kawarai T, Arawaka S, Supala A, Song YQ, Rogaeva E, Holmes E, Zhang DM, Milman P, Fraser P, Haass C, St George-Hyslop P (2000) Mutation of conserved aspartates affect maturation of presenilin 1 and presenilin 2 complexes. Acta Neurol Scand Suppl 176:6-11.

Yu H, Saura CA, Choi SY, Sun LD, Yang X, Handler M, Kawarabayashi T, Younkin L, Fedeles B, Wilson MA, Younkin S, Kandel ER, Kirkwood A, Shen J (2001) APP processing and synaptic plasticity in presenilin-1 conditional knockout mice. Neuron 31:713-726.

Zhang H, Sun S, Herreman A, De Strooper B, Bezprozvanny I (2010) Role of presenilins in neuronal calcium homeostasis. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:8566-8580.

Zhang J, Kang DE, Xia W, Okochi M, Mori H, Selkoe DJ, Koo EH (1998) Subcellular distribution and turnover of presenilins in transfected cells. J Biol Chem 273:12436-12442.

Zhang M, Haapasalo A, Kim DY, Ingano LA, Pettingell WH, Kovacs DM (2006) Presenilin/gamma-secretase activity regulates protein clearance from the endocytic recycling compartment. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 20:1176-1178.

Zhang PJ, Zhao J, Li HY, Man JH, He K, Zhou T, Pan X, Li AL, Gong WL, Jin BF, Xia Q, Yu M, Shen BF, Zhang XM (2007) CUE domain containing 2 regulates degradation of progesterone receptor by ubiquitin-proteasome. Embo J 26:1831-1842.

Zhang YW, Luo WJ, Wang H, Lin P, Vetrivel KS, Liao F, Li F, Wong PC, Farquhar MG, Thinakaran G, Xu H (2005) Nicastrin is critical for stability and trafficking but not association of other presenilin/gamma-secretase components. The Journal of biological chemistry 280:17020-17026.

Zou K, Hosono T, Nakamura T, Shiraishi H, Maeda T, Komano H, Yanagisawa K, Michikawa M (2008) Novel role of presenilins in maturation and transport of integrin beta 1. Biochemistry 47:3370-3378.

Zucchelli S, Marcuzzi F, Codrich M, Agostoni E, Vilotti S, Biagioli M, Pinto M, Carnemolla A, Santoro C, Gustincich S, Persichetti F (2011) Tumor Necrosis factor receptor associated factor 6 (TRAF6) associates with huntingtin protein and promotes its atypical ubiquitination to enhance aggregate formation. The Journal of biological chemistry.

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